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(54) Title: PURIFIED pH NEUTRAL RHIZOCTONIA LACCASES AND NUCLEIC ACIDS ENCODING SAME

(57) Abstract

The present invention relates to isolated nucleic acid fragments containing a sequence encoding a *Rhizoctonia solani* laccase having optimum activity at a neutral or basic pH, and the laccase proteins encoded thereby.

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PURIFIED PH NEUTRAL RHIZOCTONIA LACCASES AND NUCLEIC ACIDS ENCODING SAME

Related Applications

This application is a continuation-in-part of co-pending U.S. Serial Nos. 08/122,230, 08/122,827, and 08/162,827, the contents of which are incorporated by reference in their entirety.

Field of the Invention

The present invention relates to isolated nucleic acid fragments encoding a fungal oxidoreductase enzyme and the purified enzymes produced thereby. More particularly, the invention relates to nucleic acid fragments encoding a phenol oxidase, specifically a laccase, which functions at a neutral pH.

Background of the Invention

Laccases (benzenediol:oxygen oxidoreductases) are multi-copper containing enzymes that catalyze the oxidation of phenolics. Laccase-mediated oxidations result in the production of aryloxy-radical intermediates from suitable phenolic substrate; the ultimate coupling of the intermediates so produced provides a combination of dimeric, oligomeric, and polymeric reaction products. Such reactions are important in nature in biosynthetic pathways which lead to the formation of melanin, alkaloids, toxins, lignins, and humic acids. Laccases are produced by a wide variety of fungi, including ascomycetes such as *Aspergillus, Neurospora,* and *Podospora,* the deuteromycete *Botrytis*, and

basidiomycetes such as *Collybia, Fomes, Lentinus, Pleurotus, Trametes,* and perfect forms of *Rhizoctonia*. Laccase exhibits a wide range of substrate specificity, and each different fungal laccase usually differs only quantitatively from others in its ability to oxidize phenolic substrates. Because of the substrate diversity, laccases generally have found many potential industrial applications. Among these are lignin modification, paper strengthening, dye transfer inhibition in detergents, phenol polymerization, juice manufacture, phenol resin production, and waste water treatment.

Although the catalytic capabilities are similar, laccases made by different fungal species do have different temperature and pH optima, and these may also differ depending on the specific substrate. A number of these fungal laccases have been isolated, and the genes for several of these have been cloned. For example, Choi *et al.*(Mol. Plant-Microbe Interactions 5: 119-128, 1992) describe the molecular characterization and cloning of the gene encoding the laccase of the chestnut blight fungus, *Cryphonectria parasitica*. Kojima *et al.* (J. Biol. Chem. 265: 15224-15230, 1990; JP 2-238885) provide a description of two allelic forms of the laccase of the white-rot basidiomycete *Coriolus hirsutus*. Germann and Lerch (Experientia 41: 801,1985; PNAS USA 83: 8854-8858, 1986) have reported the cloning and partial sequencing of the *Neurospora crassa* laccase gene. Saloheimo *et al.*(J. Gen. Microbiol. 137: 1537-1544, 1985; WO 92/01046) have disclosed a structural analysis of the laccase gene from the fungus *Phlebia radiata*. However, virtually all of the known fungal laccases function best at acidic pHs (e.g., between pH 3.0 and 6.0), and are typically inactive at

neutral or basic pHs. Since a number of the aforestated potential industrial methods are preferentially conducted at neutral or basic pH, most fungal laccases perform poorly in such methods. Thus, the available fungal laccases are inadequate for application in a number of important commercial methods.

An exception to this rule is the extracellular laccase produced by certain species of *Rhizoctonia*. Bollag *et al.* have reported a laccase with a pH optimum of about 7.0 produced by *Rhizoctonia praticola*. A laccase of this type would be far more useful in industrial methods requiring neutral pH than previously known laccases. However, the *R. praticola* enzyme was neither purified nor further characterized, nor,to date, has any other laccase having this trait been purified or characterized. Moreover, although other laccase genes have been isolated, as described above, these have been genes encoding enzymes which function best at acidic pH. Recombinant production and commercially adequate yields of a pH neutral or basic laccase have thus been unattainable due to the fact that neither the enzyme per se nor the laccase gene encoding such an enzyme has previously been isolated and/or purified and sequenced. The present invention now provides a solution to each of these problems.

Summary of the Invention

The present invention relates to an isolated nucleic acid fragment comprising a nucleic acid sequence encoding a *Rhizoctonia* laccase which functions optimally at a pH between 6.0 to 8.5. By "functioning optimally" is meant that the enzyme exhibits significant(i.e., at least about 30% of maximum, preferably at least about 50%, and most

preferably from 50% to maximum) activity within the pH range of between about 6.0-8.5, as determined by activity in one or more standard laccase assays for substrates such as the syringaldazine, ABTS, 2,6-dimethoxyphenol, or 4 antiaminopyrine + N-ethyl-N-sulfobutyl-m-toluidine. A preferred substrate for the laccases of the present invention is syringaldazine. In a preferred embodiment, the laccase is a *Rhizoctonia solani* laccase. The invention also relates to a substantially pure laccase encoded by the novel nucleic acid sequence. By "substantially pure" is meant a laccase which is essentially (i.e.,≥90%) free of other non-laccase proteins.

In order to facilitate production of the novel laccase, the invention also provides vectors and host cells comprising the claimed nucleic acid fragment, which vectors and host cells are useful in recombinant production of the laccase. The nucleic acid fragment is operably linked to transcription and translation signals capable of directing expression of the laccase protein in the host cell of choice. A preferred host cell is a fungal cell, most preferably of the genus *Aspergillus*. Recombinant production of the laccase of the invention is achieved by culturing a host cell transformed or transfected with the nucleic acid fragment of the invention, or progeny thereof, under conditions suitable for expression of the laccase protein, and recovering the laccase protein from the culture.

The laccases of the present invention are useful in a number of industrial processes in which oxidation of phenolics is required. These processes include lignin manipulation, juice manufacture, phenol polymerization and phenol resin production. In a preferred embodiment, the

enzyme of the invention is used in a process requiring a neutral or somewhat basic pH for greatest efficiency.

Brief Description of the Figures

Figure 1 illustrates the nucleotide and amino acid sequence of RSlac1. Lower case letters in the nucleotide sequence indicate the position of introns.

Figure 2 illustrates the nucleotide and amino acid sequence of RSlac2. Lower case letters in the nucleotide sequence indicate the position of introns.

Figure 3 illustrates a restriction map of the plasmid pMWR-1.

Figure 4 illustrates the nucleotide and amino acid sequence of the translated region of RSlac3.

Figure 5 illustrates the syringaldazine oxidase activity of RSlac1 (90mM buffer, 20 µM syringaldazine, 20°C).

Figure 6 illustrates the syringaldazine oxidase activity of RSlac2 (93mM buffer, 20 µM syringaldazine, 20°C).

Detailed Description of the Invention

Certain species of the genus *Rhizoctonia* have been reported as producing laccase; therefore, an initial search focused on identifying the presence of these enzymes in various *Rhizoctonia solani* isolates. Samples are cultured and the supernatants periodically analyzed for the presence of laccase by the ABTS method, described below. Laccase is observed in all the *Rhizoctonia* cultures. Harvested laccases are electrophoretically separated and stained with ABTS. One isolate, RS22, produces a laccase with a basic pI, and is selected for further study.

The remaining studies focus on purification and characterization of the enzyme from RS22. Briefly, the fermentation broth is filtered and concentrated by UF with a membrane cut off of about 10,000. A first ion exchange chromatography step is conducted at pH 4.5 in acetate buffer, with step elution using NaCl. The eluate is then ultrafiltered and rechromatographed, and eluted with a NaCl gradient. Active fractions are pooled for further study.

The intact protein thus isolated and purified (hereinafter referred to as RSlac3) is first subjected to partial sequencing, and the N-terminal sequence obtained is as follows:

AVRNYKFDIKNVNVAPDGFQRPIVSV (SEQ. ID. NO.: 5)

The protein is further subjected to digestion with a lysine- or glutamic-acid specific protease, and additional peptides obtained from the protein have the following sequences, which can be aligned with sequences in *Coriolus hirsutus*:

Peptide 1:

SQYVDGLRGPLVIYDPDDDH (SEQ. ID. NO: 6)

Peptide 2:

GLALVFAEAPSQIRQGVQSVQPDDA (SEQ. ID. NO.: 7)

Peptide 3:

SRYBVBBASTVVMLEBWYHTPAXVLE (SEQ. ID. NO. 8)

Peptide 4:

SLGPTPNYVNPXIRDVVRVGGTTVV (SEQ. ID. NO. 9)

The following peptides are also found, but do not correspond to *Coriolus* sequences

Peptide 5:

IRYVGGPAVX(N?)RSVI (SEQ. ID. NO.: 10)

Peptide 6:

ILANPA (SEQ. ID. NO.: 11)

Peptide 7:

YEAPSLPT (SEQ. ID. NO.: 12)

In the above sequences, B designates a residue which is either aspartic acid or asparagine, and X designates unidentified residues.

In order to initiate screening for a *Rhizoctonia* laccase gene, an *R. solani* genomic library is prepared. Total DNA is partially digested with restriction enzyme Sau3A, and electrophoresed in an agarose gel to isolate DNA fragments between 8 and 21 kb in size. The fractionated fragments are ligated to λ phage EMBL3 arms with BamHI ends, and the resulting phage packaged *in vitro*. These phage are used as a library to create a library of 170,000 plaques in *E. coli* and amplified 100-fold for future use.

In order to develop probes for isolation of the *R. solani* laccase gene, the protein sequences of five known laccases are analyzed to determine consensus sequences, and two degenerate oligonucleotides constructed based on observed consensus sequences (Choi et al. *supra*; Germann and Lerch, *supra*; Saloheimo et al, *supra*, Kojima et al, *supra*). These oligos are mixed with *R. solani* genomic DNA and a DNA fragment of 220 nucleotide fragment is amplified using a taq polymerase chain reaction(PCR). The 220-nucleotide fragment is then cloned into plasmid vector.

The PCR fragment is used as a probe to screen 25,000 plaques from the amplified genomic library. Positive clones from this screen fall into two classes that are subsequently shown, by DNA sequence analysis, to code for two different laccase genes, RS*lac1* and RS*lac2*. The nucleotide sequence for each of these genes (SEQ ID. NOS.: 1 and 3), and the predicted amino acid sequence for each protein (SEQ. ID. NOS.: 2 and 4), are presented in, respectively, Figures 1

and 2. The homology between the two sequences is approximately 63%. Compared to known laccase sequences from *Coriolus hirsutus*, *Phlebia radiata*, *Aspergillus nidulans*, *Cryphonectria parasitica*, and *Neurospora crassa*, the RS laccases show between about 30-40% homology. Each of the two coding sequences is cloned into an expression vector operably linked to *Aspergillus oryzae* taka-amylase transcription and translation signals (See Figure 3). Each of the two laccase expression vectors is transformed into an *Aspergillus oryzae* and *Aspergillus niger* host cell, and the host cells screened for the presence of laccase.

For isolation of the RS*lac*3 gene, polyA RNA is purified from *R. solani* mycelia grown in the presence of anisidine. The RNA is used as a template for cDNA synthesis. The cDNA is fractionated and fragments between 1.7-3.5 kb collected, and a cDNA library created by cloning the fractionated DNA into a yeast vector. 3000 transformants from this library are screened on ABTS. After 24 hours, a single colony appears positive. The plasmid from the colony is isolated and the insert sequenced. Portions of the predicted amino acid sequence correspond with the sequences of the fragments obtained from RS 22, described *supra*. The complete nucleotide and amino acid sequences are depicted in Figure 4, and in SEQ. ID. NOS.: 13 and 14, respectively. RS*lac3* shows 48% homology with RS*lac1* and 50% homology with RS*lac2*. RS*lac3* also shows 48% homology with the *Coriolus hirsutus* laccase gene.

According to the invention, a *Rhizoctonia* gene encoding a pH neutral or basic laccase can be obtained by methods described above, or any alternative methods known in the art, using the information provided herein. The gene can be expressed, in active form, using an expression

vector. A useful expression vector contains an element that permits stable integration of the vector into the host cell genome or autonomous replication of the vector in a host cell independent of the genome of the host cell, and preferably one or more phenotypic markers which permit easy selection of transformed host cells. The expression vector may also include control sequences encoding a promoter, ribosome binding site, translation initiation signal, and, optionally, a repressor gene or various activator genes. To permit the secretion of the expressed protein, nucleotides encoding a signal sequence may be inserted prior to the coding sequence of the gene. For expression under the direction of control sequences, a laccase gene to be treated according to the invention is operably linked to the control sequences in the proper reading frame. Promoter sequences that can be incorporated into plasmid vectors, and which can direct the transcription of the laccase gene, include but are not limited to the prokaryotic ß-lactamase promoter (Villa-Kamaroff, et al., 1978, Proc. Natl. Acad. Sci. U.S.A. 75:3727-3731) and the tac promoter (DeBoer, et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:21-25). Further references can also be found in "Useful proteins from recombinant bacteria" in Scientific American, 1980, 242:74-94; and in Sambrook et al., Molecular Cloning, 1989.

The expression vector carrying the DNA construct of the invention may be any vector which may conveniently be subjected to recombinant DNA procedures, and the choice of vector will typically depend on the host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e. a vector which exists as an extrachromosomal entity, the replication of which is

independent of chromosomal replication, e.g. a plasmid, or an extrachromosomal element, minichromosome or an artificial chromosome. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

In the vector, the DNA sequence should be operably connected to a suitable promoter sequence. The promoter may be any DNA sequence which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell. Examples of suitable promoters for directing the transcription of the DNA construct of the invention, especially in a bacterial host, are the promoter of the *lac* operon of *E.coli*, the *Streptomyces coelicolor* agarase gene *dag*A promoters, the promoters of the *Bacillus licheniformis* α-amylase gene (*amy*L), the promoters of the *Bacillus stearothermophilus* maltogenic amylase gene (*amy*M), the promoters of the *Bacillus amyloliquefaciens* α-amylase (*amy*Q), or the promoters of the *Bacillus subtilis* xylA and xylB genes. In a yeast host, a useful promoter is the eno-1 promoter. For transcription in a fungal host, examples of useful promoters are those derived from the gene encoding *A. oryzae* TAKA amylase, *Rhizomucor miehei* aspartic proteinase, *A. niger* neutral α-amylase, *A. niger* acid stable α-amylase, *A. niger* or *A. awamsii* glucoamylase (gluA), *Rhizomucor miehei* lipase, *A. oryzae* alkaline protease, *A. oryzae* triose phosphate isomerase or *A. nidulans* acetamidase. Preferred are the TAKA-amylase and gluA promoters.

The expression vector of the invention may also comprise a suitable transcription terminator and, in eukaryotes, polyadenylation sequences operably connected to the DNA sequence encoding the laccase of the invention. Termination and polyadenylation sequences may suitably be derived from the same sources as the promoter.The vector may further comprise a DNA sequence enabling the vector to replicate in the host cell in question. Examples of such sequences are the origins of replication of plasmids pUC19, pACYC177, pUB110, pE194, pAMB1 and pIJ702.

The vector may also comprise a selectable marker, e.g. a gene the product of which complements a defect in the host cell, such as the *dal* genes from *B.subtilis* or *B.licheniformis*, or one which confers antibiotic resistance such as ampicillin, kanamycin, chloramphenicol or tetracycline resistance. Examples of *Aspergillus* selection markers include *amdS, pyrG, argB, niaD* and *sC*, a marker giving rise to hygromycin resistance. Preferred for use in an *Aspergillus* host cell are the *amdS* and *pyrG* markers of *A. nidulans* or *A. oryzae*. A frequently used mammalian marker is the dihydrofolate reductase (DHFR) gene. Furthermore, selection may be accomplished by co-transformation, e.g. as described in WO 91/17243.

It is generally preferred that the expression is extracellular. The laccases of the present invention may thus comprise a preregion permitting secretion of the expressed protein into the culture medium. If desirable, this preregion may be native to the laccase of the invention or substituted with a different preregion or signal sequence, conveniently accomplished by substitution of the

DNA sequences encoding the respective preregions. For example, the preregion may be derived from a glucoamylase or an amylase gene from an *Aspergillus* species, an amylase gene from a *Bacillus* species, a lipase or proteinase gene from *Rhizomucor miehei*, the gene for the α-factor from *Saccharomyces cerevisiae* or the calf prochymosin gene. Particularly preferred, when the host is a fungal cell, is the preregion for *A. oryzae* TAKA amylase, *A. niger* neutral amylase, the maltogenic amylase form *Bacillus* NCIB 11837, *B. stearothermophilus* α-amylase, or *Bacillus licheniformis* subtilisin. An effective signal sequence is the *A. oryzae* TAKA amylase signal, the *Rhizomucor miehei* aspartic proteinase signal and the *Rhizomucor miehei* lipase signal.

The procedures used to ligate the DNA construct of the invention, the promoter, terminator and other elements, respectively, and to insert them into suitable vectors containing the information necessary for replication, are well known to persons skilled in the art (cf., for instance, Sambrook et al. Molecular Cloning, 1989).

The cell of the invention either comprising a DNA construct or an expression vector of the invention as defined above is advantageously used as a host cell in the recombinant production of a enzyme of the invention. The cell may be transformed with the DNA construct of the invention, conveniently by integrating the DNA construct in the host chromosome. This integration is generally considered to be an advantage as the DNA sequence is more likely to be stably maintained in the cell. Integration of the DNA constructs into the host chromosome may be performed

according to conventional methods, e.g. by homologous or heterologous recombination. Alternatively, the cell may be transformed with an expression vector as described above in connection with the different types of host cells.

The host cell may be selected from prokaryotic cells, such as bacterial cells. Examples of suitable bacteria are gram positive bacteria such as *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus lentus*, *Bacillus brevis*, *Bacillus stearothermophilus*, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus coagulans*, *Bacillus circulans*, *Bacillus lautus*, *Bacillus megaterium*, *Bacillus thuringiensis*, or *Streptomyces lividans* or *Streptomyces murinus*, or gram negative bacteria such as *E.coli*. The transformation of the bacteria may for instance be effected by protoplast transformation or by using competent cells in a manner known *per se*.

The host cell may also be a eukaryote, such as mammalian cells, insect cells, plant cells or preferably fungal cells, including yeast and filamentous fungi. For example, useful mammalian cells include CHO or COS cells. A yeast host cell may be selected from a species of *Saccharomyces* or *Schizosaccharomyces*, e.g. *Saccharomyces cerevisiae*. Useful filamentous fungi may selected from a species of *Aspergillus*, e.g. *Aspergillus oryzae* or *Aspergillus niger*. Alternatively, a strain of a *Fusarium* species, e.g. *F. oxysporum*, can be used as a host cell. Fungal cells may be transformed by a process involving protoplast formation and transformation of the protoplasts followed by regeneration of the cell wall in a manner known *per se*. A suitable procedure for transformation of *Aspergillus* host cells is described in EP 238 023. A suitable method of

transforming *Fusarium* species is described by Malardier et al., 1989.

The present invention thus provides a method of producing a recombinant laccase of the invention, which method comprises cultivating a host cell as described above under conditions conducive to the production of the enzyme and recovering the enzyme from the cells and/or culture medium. The medium used to cultivate the cells may be any conventional medium suitable for growing the host cell in question and obtaining expression of the laccase of the invention. Suitable media are available from commercial suppliers or may be prepared according to published formulae (e.g. in catalogues of the American Type Culture Collection).

The resulting enzyme may be recovered from the medium by conventional procedures including separating the cells from the medium by centrifugation or filtration, precipitating the proteinaceous components of the supernatant or filtrate by means of a salt, e.g. ammonium sulphate, followed by purification by a variety of chromatographic procedures, e.g. ion exchange chromatography, gel filtration chromatography, affinity chromatography, or the like. Preferably, the isolated protein is about 90% pure as determined by SDS-PAGE, purity being most important in food, juice or detergent applications.

In a particularly preferred embodiment, the expression of laccase is achieved in a fungal host cell, such as *Aspergillus*. As described in detail in the following examples, the laccase gene is ligated into a plasmid containing the *Aspergillus oryzae* TAKA α-amylase promoter, and the *Aspergillus nidulans amdS* selectable marker. Alternatively, the *amdS* may be on a separate plasmid and

used in co-transformation. The plasmid (or plasmids) is used to transform an *Aspergillus* species host cell, such as *A. oryzae* or *A. niger* in accordance with methods described in Yelton et al. (PNAS USA 81: 1470-1474,1984).

Those skilled in the art will recognize that the invention is not limited to use of the nucleic acid fragments specifically disclosed herein, for example, in Figures 1 and 2. It will be apparent that the invention also encompasses those nucleotide sequences that encode the same amino acid sequences as depicted in Figures 1, 2 and 3, but which differ from those specifically depicted nucleotide sequences by virtue of the degeneracy of the genetic code. In addition, the invention also encompasses other nucleotide fragments,and the proteins encoded thereby, which encode laccase proteins having substantially the same pH optimum as those of *Rhizoctonia solani,* and which show a significant level of homology with the *Rhizoctonia solani* amino acid sequence. For example, the present data show that more than one species of *Rhizoctonia* produces a laccase with the desired pH profile; it is therefore expected that other *Rhizoctonia* species also produce similar laccases and therefore, using the technology described herein, can be used as a source for genes within the scope of the claimed invention. As also shown in the present examples, not only is there more than one nucleotide and amino acid sequence that encodes a laccase with the required characteristics, there is also considerable variation tolerated within the sequence while still producing a functional enzyme. Therefore, the invention also encompasses any variant nucleotide sequence, and the protein encoded thereby, which protein retains at least about an 80% homology with one or the other of the amino acid sequences depicted in Figures 1,

2 and 3, and retains both the laccase and pH optimum activity of the sequences described herein. In particular, variants which retain a high level(i.e., ≥ 80%) of homology at highly conserved regions of the *Rhizoctonia* laccase are contemplated. Such regions are identified as residues 458-469 in RSLAC1, and 478-489 in RSLAC2; and residues 131-144 in RSLACI and 132-145 in RSLAC2.

Useful variants within the categories defined above include, for example, ones in which conservative amino acid substitutions have been made, which substitutions do not significantly affect the activity of the protein. By conservative substitution is meant that amino acids of the same class may be substituted by any other of that class. For example, the nonpolar aliphatic residues Ala, Val, Leu, and Ile may be interchanged, as may be the basic residues Lys and Arg, or the acidic residues Asp and Glu. Similarly, Ser and Thr are conservative substitutions for each other, as are Asn and Gln. It will be apparent to the skilled artisan that such substitutions can be made outside the regions critical to the function of the molecule and still result in an active enzyme. Retention of the desired activity can readily be determined by conducting a standard ABTS oxidation method in 0.1M sodium phosphate at pH 7.0.

The protein can be used in number of different industrial processes; although the enzyme is also functional to some extent at lower pH, the *R. solani* laccase is most beneficially used in processes that are usually conducted at a neutral or alkaline pH, since other laccases are not active in this pH range. These processes include polymerization of lignin, both Kraft and lignosulfates, in solution, in order to produce a lignin with a higher molecular weight. A neutral/alkaline laccase is a

particular advantage in that Kraft lignin is more soluble at higher pHs. Such methods are described in, for example, Jin et al., Holzforschung 45(6): 467-468, 1991; US Patent No. 4,432,921; EP 0 275 544; PCT/DK93/00217, 1992.

The laccase of the present invention can also be used for in-situ depolymerization of lignin in Kraft pulp, thereby producing a pulp with lower lignin content. This use of laccase is an improvement over the current use of chlorine for depolymerization of lignin, which leads to the production of chlorinated aromatic compounds, which are an environmentally undesirable by-product of paper mills. Such uses are described in, for example, Current opinion in Biotechnology 3: 261-266, 1992; J. Biotechnol. 25: 333-339, 1992; Hiroi et al., Svensk papperstidning 5: 162-166, 1976. Since the environment in a paper mill is typically alkaline, the present laccase is more useful for this purpose than other known laccases, which function best under acidic conditions.

Oxidation of dyes and other chromophoric compounds leads to decolorization of the compounds. Laccase can be used for this purpose, which can be particularly advantageous in a situation in which a dye transfer between fabrics is undesirable, e.g., in the textile industry and in the detergent industry. Methods for dye transfer inhibition and dye oxidation can be found in WO 92/01406, WO 92/18683, EP 0495836 and Calvo, Mededelingen van de Faculteit Landbouw-wetenschappen/Rijiksuniversitet Gent.56: 1565-1567, 1991.

The present laccase can also be used for the polymerization of phenolic compounds present in liquids. An example of such utility is the treatment of juices, such as apple juice, so that the laccase will accelerate a

precipitation of the phenolic compounds present in the juice, thereby producing a more stable juice. Such applications have been described in Stutz, Fruit processing 7/93, 248-252, 1993; Maier et al., Dt. Lebensmittel-rindschau 86(5): 137-142, 1990; Dietrich et al., Fluss. Obst 57(2): 67-73, 1990. The invention is further illustrated by the following non-limiting examples.

EXAMPLES

1. Purification and characterization of R. solani laccase

Individual isolates of *R. solani* cultured on potato dextrose agar (Difco) are examined for laccase enzyme formation by transferring a small piece of agar containing vigorous growth to 100 ml CFM (24.0 g potato dextrose broth, 3.0 g yeast extract, 1.0 ml Microelement solution [0.80 g KH_2PO_4, 0.64 g $CuSO_4 \cdot 5H_2O$, 0.11 g $FeSO_4 \cdot 7H_2O$, 0.80 g $MnCl_2 \cdot 4H_2O$, 0.15 g $ZnSO_4 \cdot 7H_2O$, distilled water to 1000 ml], distilled water to 1000 ml) in a 500 ml shake flask. Incubation is at room temperature, at 200 rpm on an orbital shaker.

Samples are harvested at 50, 74, 122 and 170 hours, centrifuged and the clear supernatant analyzed for laccase with its ABTS (ABTS= 2,2'-azinobis (3 ethylbenzothiazoline-6-sulfonic acid). The analysis is carried out by adding 200 µl of 2mM ABTS in 0.1 M phosphate buffer, pH 7, and observing the change in absorbance at 418 nm after 30 minutes incubation at room temperature (approximately 23-25° C). This method is modified from a peroxidase analysis method described by Pütter and Becker (Peroxidases, in: Bergmeyer, H.U. (ed.), Methods of Enzymatic Analysis, 3rd ed., Vol.III, pp.286-293, 1983)

Each of the laccases harvested at 172 hours is electrophoretically separated and stained with ABTS as

chromogen. Several distinct patterns emerge; the strain RS 22 is shown to produce a laccase having a basic pI, and is chosen for further characterization.

Laccase activity is also determinable by the syringaldazine method. Laccase catalyzes the oxidation of syringaldazine to tetramethoxy azo bis-methylene quinone under aerobic conditions, with a change of color from yellow to violet. 3000 µl of 25 mM acetate buffer (containing 10mg/l cuprisulfate, 5 H_2O) at pH 5.5, 30°C, is mixed in a 1 cm cuvette with 225 µl 0.28 mM syringaldazine (5mg solubilized in 25 ml ethanol and adjusted to 50 ml with demineralized water). The mixture is then mixed with 100 µl of a laccase dilution (diluted in acetate buffer so that the increase in absorbance(ΔOD) is within the range of 0.1-0.6). The reaction mixture is placed in a 30°C thermostated spectrophotometer and the reaction is followed at 530 nm for 10 to 70 seconds from the addition of laccase. The activity of the enzyme is calculated as ΔOD/minute x 0.677 x dilution factor, and is expressed as LACU.

For purification of the *Rhizoctonia* laccase, 2.1 liter of culture medium with a LACU activity of 0.19 LACU/ml is filtered through a 10 µm filter and concentrated to 230 ml by ultrafiltration using a Filtron Minisette OMEGA membrane with a cutoff value of 10 kDa. The pH of the sample is 5.3 and the activity of the concentrated sample is determined to be 3.34 LACU/ml.

After pH adjustment to 4.5 and filtration due to slight precipitation, the sample is applied to a 40 ml S Sepharose Fast Flow column equilibrated with 20mM acetate buffer at pH 4.5 (buffer A). The column is washed in buffer A and eluted with buffer A containing 1 M NaCl. Active fractions are collected and pooled. This active pool is concentrated and

buffer exchanged to buffer A using an Amicon ultrafiltration unit equipped with a Diaflo YM10 membrane. This sample is rechromatographed on a 5 ml S Sepharose High Performance column using the method described above except that elution is carried out with a linear gradient over 30 column volumes from buffer A to buffer A containing 0.5 M NaCl. The fractions from this purification exhibiting highest activity are pooled. Approximately 45 mg laccase are obtained, when protein concentration is estimated by one absorption unit at A280 nm equal to 1mg/ml. The protein is >90% pure as judged by SDS-PAGE. The molecular weight estimated by SDS-PAGE is approximately 67 kDa. The specific activity of the purified protein is 1 LACU/mg. The pH profile of the purified protein, using syringaldazine as substrate is show in Table 1, below.

Table 1.

pH	5	6	7	8
% activity	0.5	31	100	59

For sequencing of the protein, peptides are generated using wither a lysine-specific protease from *Achromobacter* (*Achromobacter* protease I) or a glutamic acid specific protease from *Bacillus licheniformes*. The peptides are purified by reverse phase HPLC employing linear gradients of 80% 2-propanol containing 0.08% aqueous TFA (solvent B) in 0.1% aqueous TFA (solvent A).

N-terminal amino acid sequence analysis of the intact protein and of purified peptides are carried out in an Applied Biosystems 473A protein sequencer according to the manufacturer's instructions. Initial partial sequencing of

the isolated protein yields the following N-terminal sequence:

AVRNYKFDIKNVNVAPDGFQRPIVSV (SEQ. ID. NO.: 5)

The protein is then digested with either a lysine- or glutamic-acid specific protease, and following additional peptides identified. Peptides 1-4 can be aligned with sequences in the laccase of *Coriolus hirsutus*:

Peptide 1:

SQYVDGLRGPLVIYDPDDDH (SEQ. ID. NO: 6)

Peptide 2:

GLALVFAEAPSQIRQGVQSVQPDDA (SEQ. ID. NO.: 7)

Peptide 3:

SRYBVBBASTVVMLEBWYHTPAXVLE (SEQ. ID. NO. 8)

Peptide 4:

SLGPTPNYVNPXIRDVVRVGGTTVV (SEQ. ID. NO. 9)

Peptide 5:

IRYVGGPAVX(N?)RSVI (SEQ. ID. NO.: 10)

Peptide 6:

ILANPA (SEQ. ID. NO.: 11)

Peptide 7:

YEAPSLPT (SEQ. ID. NO.: 12)

An X in the above sequences designates an unidentified residue, and B represents a residue which is either aspartic acid or asparagine.

2. Isolation of *R. solani* laccase gene

A study of the known amino acid sequences of fungal laccases obtained from non-*Rhizoctonia* species (Choi et al., *supra*; German et al., *supra*; Saloheimo et al. *supra*; and Kojima et al, *supra*) is conducted to determine the presence of consensus sequences among them. Two regions of high identity, IHWHGFFQ and TFWYHSH, are found near the amino

terminal third of the protein. Based on these consensus sequences and the corresponding DNA sequences, three degenerate oligonucleotides, O-lac2 [TGG/AAAGACCATA/GGTGTCG/AGTA/G],its complement O-lac2r, and O-lac3[ATCCAT/CTGGCAT/CGGG/CA/TTCTTCCAG/A], are synthesized using an Applied Biosystems 394 DNA/RNA synthesizer.

The synthesized oligos are used in a polymerase chain reaction (PCR) to screen *Rhizoctonia solani* genomic DNA for a laccase gene or fragment thereof. For amplifications of genomic DNA, 0.5 µg of genomic DNA is incubated with 1µM of each primer, 200µM of dNTPs, and 1 U taq polymerase (Boehringer Mannheim) in [10 mM Tris-Cl, 1.5 mM $MgCl_2$, 50 mM KCl, 1 mg/ml gelatine;pH 8.3]. The reactions are incubated for 1x5 minutes at 95°C, 30x[1 minute at 95°C, 1 minute at 50-60°C, 1 minute at 72°C], and 1x5 minutes at 72°C. The PCR reactions amplify a DNA fragment of 220 nucleotides. The PCR product is cloned, according to manufacturer's directions, into the TA cloning vector (InVitrogen Corp.). Characterization of the PCR product by DNA sequencing of individual clones distinguishes two separate laccase genes designated RS*lac*1 and RS*lac*2.

To prepare a *R. solani* genomic library, *R. solani* DNA is partially digested with restriction enzyme Sau3A, and electrophoresed through a 0.8% Sea Plaque Agarose (FMC Bioproducts) in a Tris/Acetate/EDTA buffer to isolate those DNA fragments between 8.0 an 21 kb in size. The gel fractionated fragments are further purified with Beta-Agarase(New England Biolabs) according to manufacturer's instruction, and then ligated to lambda phage EMBL3 arms with BamHI ends. The resulting phages are packaged in vitro using Gigapack II packaging extract(Stratagene). 25 ml of TB media+0.2% maltose and 10 $MgSO_4$ is inoculated into a 50 µl

aliquot of an overnight culture of *E. coli* K802 (supE, hsdR, gal, metB) and incubated at 37°C with shaking until the A600=0.5. 25 µl of a 1:10 and 1:50 dilution of the packaged phage are mixed with 250 µl of the K802 cells, and incubated for 20 minutes at 37°C. To each dilution, 5 µl of melted top agar at 48°C are added. The mix is then plated onto prewarmed LB plates and incubated at 37°C for at least 12 hours. From these phage, a library of 170,000 plaques in *E.coli* K802 is created and amplified 100-fold for future use.

To screen for the laccase gene, 25,000 plaques from the amplified genomic library are plated onto NZY/agarose plates for plaque lifts using conventional methods. Filters are screened using the 220 nucleotide PCR fragment randomly labelled to $5x10^8$ cpm/µg as a probe. Filters are hybridized in 50% formamide, 6xSSC for 16 hours at 42°C and washed with 0.5xSSC, 0.1% SDS at 65°C. Positive clones are picked and rescreened using conventional methods. The nine positive clones identified fell into two classes that by DNA sequence analysis are shown to code for two different laccase genes, RS*lac*1 and RS*lac*2. The complete nucleotide sequence of each of these genes is determined using fluorescent nucleotides and an Applied Biosystems automatic DNA sequencer (Model 363A, version 1.2.0). The nucleotide and predicted amino acid sequences are depicted in Figures 1 and 2.

For isolation of RS*lac*3, poly A RNA purified from *R. solani* mycelia grown in the presence of 1 mM anisidine is used as a template for cDNA synthesis using standard protocols. The cDNA is fractionated by electrophoresis through a 0.8% agarose gel and DNA fragments between 1.7 and 3.5 kb in size are collected. A library is then created by cloning the size-fractionated cDNA into the yeast expression

vector pYES2. 3000 yeast transformants from this library are plated initially on YNB (1.7 g yeast nitrogen base without amino acids, 5 g $(NH_4)_2SO_4$ per liter) with 2% glucose. After 4 days growth at 30°C, the resulting colonies are replica plated to YNB with 0.1% glucose, 2% galactose and 2mM ABTS [2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid; Sigma # A-1888). After 24 hours of growth at 30°C a single colony has a light green halo which gradually turns a dark purple. The plasmid from this colony is isolated and the insert sequenced. The sequence of the translated portion of the RS*lac*3 gene and protein is shown in SEQ.ID NOS. 13 and 14, and in Figure 4.

<u>3. Expression of laccase gene</u>

The plasmid pMWR-1 is a pUC derived vector containing the TAKA amylase transcription regulation signals and the TAKA amylase signal sequence. This plasmid is engineered with a unique SfiI site at the signal sequence cleavage site, and a 3' adjacent NsiI site such that these two restriction enzymes can be used to introduce, in frame, a foreign protein. Using a PCR reaction (conducted as described above, but with 100 ng of the appropriate linearized plasmid DNA as a template) and mutagenized primers, an SfiI site is introduced at amino acid 12 and amino acid 14 of RSlac1 and RSlac2, respectively, such that the protein coding sequences are in frame with the TAKA signal sequence. In addition, a PCR amplification is also used to introduce a PstI site (CTGCAG) at the 3' end of RS*lac*1 and an NsiI site (ATGCAT) at the 3' end of RS*lac*2.

To prepare for transformation, cells of *Aspergillus oryzae* are cultivated in YPG (1g/l yeast extract, 0.25 g K_2PO_4. 0.125 g/$MgSO_4$, 3.75 g glucose) at 34°C with 100-120rpm

for 16-20 hours, then collected by filtration with miracloth. Cells are washed with Mg solution (0.6M $MgSO_4 \cdot 7H_2O$), then 2-6 g of cells are taken up in 10 ml MgP(1.2M $MgSO_4 \cdot 7H_2O$, 10mM $NaH_2PO_4 \cdot 2H_2O$;pH 5.8).To this is added 1 ml of Novozyme® 234 (120 mg/ml MgP), and the sample kept on ice for 5 minutes. One ml of BSA (12 mg/ml) is added, and the sample shaken gently at 34-37°C. Protoplasts are collected by filtration through miracloth, and overlain with 5 ml of ST (0.6 M Sorbitol, 100mM Tris; pH 7). The sample is spun at 2500 rpm for 15 minutes, and a band of protoplasts collected. Two volumes of STC (1.2M Sorbitol, 10 mM tris, 10 mM $CaCl_2 \cdot 2H_2O$;pH 7.5) are added and the sample is spun at 2500 rpm for 5 minutes. The precipitate is washed twice with 5 ml of STC, and the protoplasts suspended in 0.5-1ml of STC.

For the transformation process, the protoplast concentration is adjusted to $1\text{-}5 \times 10^7$/ml. To 100 µl of protoplast solution is added a maximum of 10 µl of DNA solution (5-10 µg of supercoiled DNA) and 0.2 ml of PEG (60% PEG4000, 10mM Tris, 10mM $CaCl_2 \cdot H_2O$; pH 7.5),and the combination is mixed well. The sample is kept at room temperature for 25 minutes; then to it is added first 0.2 ml PEG, with mixing, the 0.85 ml PEG with mixing. The mixture is kept at room temperature for 20 minutes, then spun at 4000 rpm for 15 minutes. The precipitate is washed with 2 ml of STC by spinning at 2500 rpm for 10 minutes. The protoplasts are resuspended in 0.2-0.5 ml of STC, and then spread on COVE plates. COVE medium (pH 7) contains 342.3 g/l sucrose, 25 g/l agar and a salt solution comprising 26 g/l KCl, 26 g/l $MgSO_4 \cdot H_2O$, 76 g/l KH_2PO_4, and 50 ml/l of trace metals; the trace metals are 40 mg/l $NaB_4O_7 \cdot 10H_2O$, 400 mg/l

$CuSO_4 \cdot 5H_2O$, 1200mg/l $FeSO_4 \cdot 7H_2O$, 700mg/l $MnSO_4 \cdot H_2O$, 800mg/l $Na_2MoO_2 \cdot 2H_2O$, 10 g/l $ZnSO_4 \cdot 7H_2O$). After autoclaving, 10 ml/l of 1M filtrated acetamide and 5-10 ml of 3M CsCl are added to the solution. Transformants are selected by growth cells on COVE medium which contains acetamide as the carbon source.

The confirmation of laccase production in the samples is determined by the ABTS oxidation method as described above on Cove medium with 2 mM ABTS, at pH 5 and 7.3. Both RS*lac*1 and RS*lac*2 express laccase activity at pH 5 and pH 7, in contrast with a control laccase which shows substantially no activity at pH 7.3.

The products of the expression of each of RS*lac*1 and RS*lac*2 are tested for oxidase activity at various pHs using syringaldazine as the substrate. The assay is conducted substantially as described above for the assay of the native protein, over pH range of 4-9. As shown in Figures 5 and 6, both laccases are active at pHs over pH 5, and RS*lac*1 has particularly good activity at pHs over 6. The pattern of activity is generally comparable to that observed for the RS*lac*3 laccase isolated from RS 22 (see Table 1 above), with RS*lac*1 exhibiting the broadest range of activity.

Deposit of Biological Materials

The following biological materials have been deposited under the terms of the Budapest Treaty in the International Mycological Institute, Genetic Resource Reference Collection, located at Bakeham Lane, Egham, Surrey TW20 9TY and given the following accession number.

Deposit	Accession Number
Rhizoctonia solani RS22	IMI CC 358730

The following biological materials have been deposited under the terms of the Budapest Treaty with the Agricultural Research Service Patent Culture Collection, Northern Regional Research Center, 1815 University Street, Peoria, Illinois, 61604 and given the following accession numbers.

Deposit	Accession Number
E. coli containing RS*lac*1 fused to an α-amylase signal sequence (EMCC 00844)	NRRL B-21141
E. coli containing RS*lac*2 with an SfiI site insert (EMCC 00845)	NRRL B-21142
E. coli containing RS*lac*3 (EMCC 0088)	NRRL B-21156

SEQUENCE LISTING

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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: to be assigned
(B) FILING DATE: 13-SEP-1994

(vii) PRIOR APPLICATION DATA:
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(B) FILING DATE: 22-DEC-1993

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(B) FILING DATE: 17-SEP-1993

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/122,827
(B) FILING DATE: 17-SEP-1993

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/162,827
(B) FILING DATE: 03-DEC-1993

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2838 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Rhizoctonia laccase

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 302..351

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 463..512

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 576..633

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 760..818

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 822..877

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 1001..1054

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 1316..1372

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 1697..1754

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 1827..1880

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(B) LOCATION: 1992..2051

(ix) FEATURE:
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(B) LOCATION: 2157..2206

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 2348..2404

(ix) FEATURE:

(A) NAME/KEY: intron
(B) LOCATION: 2438..2498

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: join(170..301, 352..462, 513..575, 634..759, 819..821, 878..1000, 1055..1315, 1373..1696, 1755..1826, 1881..1991, 2052..2156, 2207..2347, 2405..2437, 2499..2621)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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AGATTTCGAT ATCCCCTCTC GTCTCGGTTT TGGTCTCGGC TTGCCTCTA ATG GCG        175
                                                      Met Ala
                                                        1

CGC ACC ACT TTC CTT GTC TCG GTT TCG CTC TTT GTT TCC GCT GTT CTT      223
Arg Thr Thr Phe Leu Val Ser Val Ser Leu Phe Val Ser Ala Val Leu
         5                  10                  15

GCG CGC ACC GTC GAG TAC GGC TTG AAG ATT AGT GAT GGG GAG ATA GCT      271
Ala Arg Thr Val Glu Tyr Gly Leu Lys Ile Ser Asp Gly Glu Ile Ala
     20                  25                  30

CCT GAC GGT GTT AAG CGT AAT GCG ACT TTG GTACGCACTC CTTGTAATCC        321
Pro Asp Gly Val Lys Arg Asn Ala Thr Leu
 35                  40

AACAATTCAA GGTTTCTGAT GCTTGGTCAG GTA AAT GGA GGG TAT CCC GGT CCA     375
                                 Val Asn Gly Gly Tyr Pro Gly Pro
                                      45                  50

CTC ATT TTT GCC AAC AAG GGG GAT ACT CTC AAA GTC AAG GTC CAA AAC      423
Leu Ile Phe Ala Asn Lys Gly Asp Thr Leu Lys Val Lys Val Gln Asn
         55                  60                  65

AAG CTC ACG AAT CCT GAG ATG TAT CGC ACC ACT TCC ATC GTATGTTCGT       472
Lys Leu Thr Asn Pro Glu Met Tyr Arg Thr Thr Ser Ile
     70                  75                  80

TCGATATCTA CTAATACATC CGTCGCTAAA TATCTTGTAG CAT TGG CAC GGT CTC      527
                                            His Trp His Gly Leu
                                                         85

TTA CAA CAT AGA AAC GCC GAC GAC GAC GGT CCT TCG TTC GTC ACT CAG      575
Leu Gln His Arg Asn Ala Asp Asp Asp Gly Pro Ser Phe Val Thr Gln
             90                  95                 100

GTAGGATTCT GGAAGGTTGG CCTGAACTCT CTGTTAACCG ACAACCCGAT GTCACCAG      633

TGC CCG ATT GTT CCA CGC GAG TCG TAT ACT TAC ACC ATA CCT CTG GAC      681
Cys Pro Ile Val Pro Arg Glu Ser Tyr Thr Tyr Thr Ile Pro Leu Asp
        105                 110                 115

GAT CAA ACC GGA ACC TAT TGG TAC CAT AGC CAC TTG AGT TCG CAA TAC      729
Asp Gln Thr Gly Thr Tyr Trp Tyr His Ser His Leu Ser Ser Gln Tyr
    120                 125                 130

GTT GAT GGT CTT CGA GGC CCG CTG GTA ATC GTGAGTATCT TGACTTGTCT        779
Val Asp Gly Leu Arg Gly Pro Leu Val Ile
135                 140
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ACTGAAGGCA ACGAGACTAA AACAAGCGTC GATTCACAG TAT GTTCGTCTCC           831
                                          Tyr
                                          145

CCTTTATTTA GCTCTGGATC TTCATTTCTC ACGTAATACA TGATAG GAT CCC AAG      886
                                                   Asp Pro Lys

GAT CCT CAC AGG CGT TTG TAT GAT GTT GAC GAT GAG AAG ACC GTC CTG     934
Asp Pro His Arg Arg Leu Tyr Asp Val Asp Asp Glu Lys Thr Val Leu
    150                 155                 160

ATC ATC GGT GAC TGG TAT CAT GAA TCG TCC AAG GCA ATC CTT GCT TCT     982
Ile Ile Gly Asp Trp Tyr His Glu Ser Ser Lys Ala Ile Leu Ala Ser
165                 170                 175                 180

GGT AAC ATT ACC CGA CAG GTAAGTGATA CATGCCGGTC CCAGAAAAAT           1030
Gly Asn Ile Thr Arg Gln
                185

TCTCTAAATT CATTTTAATT ACAG CGA CCG GTC TCT GCC ACC ATC AAC GGC     1081
                           Arg Pro Val Ser Ala Thr Ile Asn Gly
                                       190                 195

AAA GGT CGA TTT GAC CCT GAC AAC ACT CCT GCC AAC CCA GAT ACT CTG    1129
Lys Gly Arg Phe Asp Pro Asp Asn Thr Pro Ala Asn Pro Asp Thr Leu
                200                 205                 210

TAC ACC CTC AAG GTC AAG CGA GGG AAG CGC TAT CGT CTG CGT GTC ATC    1177
Tyr Thr Leu Lys Val Lys Arg Gly Lys Arg Tyr Arg Leu Arg Val Ile
            215                 220                 225

AAT AGC TCG GAG ATC GCT TCG TTC CGA TTC AGT GTG GAA GGT CAC AAG    1225
Asn Ser Ser Glu Ile Ala Ser Phe Arg Phe Ser Val Glu Gly His Lys
        230                 235                 240

GTG ACT GTG ATT GCT GCC GAT GGC GTC TCT ACC AAA CCG TAT CAG GTC    1273
Val Thr Val Ile Ala Ala Asp Gly Val Ser Thr Lys Pro Tyr Gln Val
    245                 250                 255

GAT GCG TTT GAT ATT CTA GCA GGA CAG CGC ATA GAT TGC GTC            1315
Asp Ala Phe Asp Ile Leu Ala Gly Gln Arg Ile Asp Cys Val
260                 265                 270

GTAAGTGTCG TCCGAACCCA CATCTGAGCT CAAGTGTTGA TACATGCGCG CTTATAG     1372

GTG GAG GCG AAC CAA GAA CCC GAC ACA TAC TGG ATC AAC GCA CCG CTG    1420
Val Glu Ala Asn Gln Glu Pro Asp Thr Tyr Trp Ile Asn Ala Pro Leu
    275                 280                 285

ACC AAC GTG CCC AAC AAG ACC GCT CAG GCT CTC CTC GTT TAT GAG GAG    1468
Thr Asn Val Pro Asn Lys Thr Ala Gln Ala Leu Leu Val Tyr Glu Glu
290                 295                 300                 305

GAT CGT CGG CCG TAC CAC CCT CCA AAG GGC CCG TAT CGC AAG TGG AGC    1516
Asp Arg Arg Pro Tyr His Pro Pro Lys Gly Pro Tyr Arg Lys Trp Ser
                310                 315                 320

GTC TCT GAG GCG ATC ATC AAG TAC TGG AAT CAC AAG CAC AAG CAC GGA    1564
Val Ser Glu Ala Ile Ile Lys Tyr Trp Asn His Lys His Lys His Gly
            325                 330                 335

CGT GGT TTG CTG TCT GGA CAT GGA GGT CTC AAG GCT CGG ATG ATC GAG    1612
Arg Gly Leu Leu Ser Gly His Gly Gly Leu Lys Ala Arg Met Ile Glu
        340                 345                 350

GGT AGC CAT CAT CTG CAT TCG CGC AGC GTC GTT AAG CGC CAG AAT GAG    1660
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Gly Ser His His Leu His Ser Arg Ser Val Val Lys Arg Gln Asn Glu
    355                 360                 365

ACC ACC ACT GTT GTA ATG GAC GAG AGC AAG CTC GTT GTAAGTACCA         1706
Thr Thr Thr Val Val Met Asp Glu Ser Lys Leu Val
370                 375                 380

TATTTAAAAG TTGGTTGGGT TTCGAATACT TATTTCAACT TTTCTTAG CCA CTG GAA   1763
                                                     Pro Leu Glu

TAC CCC GGC GCT GCA TGC GGG TCT AAA CCT GCT GAC CTC GTC TTG GAT    1811
Tyr Pro Gly Ala Ala Cys Gly Ser Lys Pro Ala Asp Leu Val Leu Asp
385                 390                 395                 400

CTC ACT TTT GGT TTG GTATGTAGCC AAATCGCCCA TATACAGGAT ACTGAATATT    1866
Leu Thr Phe Gly Leu
                405

GTTTGTGCGT GTAG AAC TTT GCT ACC GGG CAC TGG ATG ATC AAC GGT ATC    1916
                Asn Phe Ala Thr Gly His Trp Met Ile Asn Gly Ile
                                410                 415

CCA TAC GAG TCT CCC AAA ATC CCC ACA TTG CTC AAG ATC CTC ACT GAT    1964
Pro Tyr Glu Ser Pro Lys Ile Pro Thr Leu Leu Lys Ile Leu Thr Asp
        420                 425                 430

GAG GAC GGG GTT ACC GAG TCT GAC TTC GTATGTTCCC TTTTCGGTAT          2011
Glu Asp Gly Val Thr Glu Ser Asp Phe
    435                 440

CTTCGTATGC GTGCACTGAC TCGTGCTGGT GGGAATTTAG ACC AAG GAG GAG CAC    2066
                                            Thr Lys Glu Glu His
                                                    445

ACA GTC ATA CTC CCG AAG AAC AAA TGC ATC GAA TTC AAC ATC AAG GGG    2114
Thr Val Ile Leu Pro Lys Asn Lys Cys Ile Glu Phe Asn Ile Lys Gly
        450                 455                 460

AAC TCG GGT ATT CCC ATT ACG CAC CCC GTA CAT CTT CAC GGT            2156
Asn Ser Gly Ile Pro Ile Thr His Pro Val His Leu His Gly
    465                 470                 475

GTAAGTGCAT ATCGGATGGT TTACGATACT AAGGCTCATC AACTTTTTAG CAC ACT     2212
                                                       His Thr

TGG GAT GTC GTA CAA TTT GGC AAC AAC CCA CCC AAT TAT GTC AAT CCT    2260
Trp Asp Val Val Gln Phe Gly Asn Asn Pro Pro Asn Tyr Val Asn Pro
480                 485                 490                 495

CCC CGT AGG GAC GTG GTT GGC TCT ACA GAT GCG GGT GTG AGG ATT CAG    2308
Pro Arg Arg Asp Val Val Gly Ser Thr Asp Ala Gly Val Arg Ile Gln
                500                 505                 510

TTC AAG ACC GAC AAT CCA GGA CCG TGG TTC CTG CAC TGC GTGCGTCGGT     2357
Phe Lys Thr Asp Asn Pro Gly Pro Trp Phe Leu His Cys
            515                 520

CCCCATCGTC CGTTATGGTT TTTCTAATAC GTCCCATTCT ATTTTAG CAT ATT GAC    2413
                                                    His Ile Asp
                                                    525

TGG CAT CTT GAG GAG GGT TTC GCA GTGAGTACTG AGACCTAAGT GCTACTCGGC   2467
Trp His Leu Glu Glu Gly Phe Ala
        530                 535
```

```
TCATTACTGA TTACCGCATG TATGCGTCTA G ATG GTG TTT GCT GAA GCG CCC       2519
                                   Met Val Phe Ala Glu Ala Pro
                                                   540

GAA GCC GTC AAG GGA GGT CCA AAG AGC GTG GCC GTG GAC TCT CAG TGG       2567
Glu Ala Val Lys Gly Gly Pro Lys Ser Val Ala Val Asp Ser Gln Trp
        545                 550                 555

GAA GGG CTG TGT GGC AAG TAC GAC AAC TGG CTA AAA TCA AAT CCG GGC       2615
Glu Gly Leu Cys Gly Lys Tyr Asp Asn Trp Leu Lys Ser Asn Pro Gly
    560                 565                 570

CAG CTG TAGGCGTATC GCAGCCACAT TGGTGATGAT TGAAAGTTGC ATCTTGTTCC       2671
Gln Leu
575

TATAACCGGC TCTTATATAC GGGTGTCTCC CAGTAAAGTC GTAGCCCAAT TTCAGCCGAG    2731

ACAGATATTT AGTGGACTCT TACTCTTGTG TCCCATTGAC GCACATCGTT GCATCAAACC    2791

TGCTTTTTAT CGTCCCTCTT TGTAATTTGT GTTGCTGTAA TGTATCG                  2838
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 576 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Met Ala Arg Thr Thr Phe Leu Val Ser Val Ser Leu Phe Val Ser Ala
  1               5                  10                  15

Val Leu Ala Arg Thr Val Glu Tyr Gly Leu Lys Ile Ser Asp Gly Glu
            20                  25                  30

Ile Ala Pro Asp Gly Val Lys Arg Asn Ala Thr Leu Val Asn Gly Gly
        35                  40                  45

Tyr Pro Gly Pro Leu Ile Phe Ala Asn Lys Gly Asp Thr Leu Lys Val
    50                  55                  60

Lys Val Gln Asn Lys Leu Thr Asn Pro Glu Met Tyr Arg Thr Thr Ser
65                  70                  75                  80

Ile His Trp His Gly Leu Leu Gln His Arg Asn Ala Asp Asp Asp Gly
                85                  90                  95

Pro Ser Phe Val Thr Gln Cys Pro Ile Val Pro Arg Glu Ser Tyr Thr
            100                 105                 110

Tyr Thr Ile Pro Leu Asp Asp Gln Thr Gly Thr Tyr Trp Tyr His Ser
        115                 120                 125

His Leu Ser Ser Gln Tyr Val Asp Gly Leu Arg Gly Pro Leu Val Ile
    130                 135                 140

Tyr Asp Pro Lys Asp Pro His Arg Arg Leu Tyr Asp Val Asp Asp Glu
145                 150                 155                 160

Lys Thr Val Leu Ile Ile Gly Asp Trp Tyr His Glu Ser Ser Lys Ala
                165                 170                 175
```

Ile Leu Ala Ser Gly Asn Ile Thr Arg Gln Arg Pro Val Ser Ala Thr
180 185 190

Ile Asn Gly Lys Gly Arg Phe Asp Pro Asp Asn Thr Pro Ala Asn Pro
195 200 205

Asp Thr Leu Tyr Thr Leu Lys Val Lys Arg Gly Lys Arg Tyr Arg Leu
210 215 220

Arg Val Ile Asn Ser Ser Glu Ile Ala Ser Phe Arg Phe Ser Val Glu
225 230 235 240

Gly His Lys Val Thr Val Ile Ala Ala Asp Gly Val Ser Thr Lys Pro
245 250 255

Tyr Gln Val Asp Ala Phe Asp Ile Leu Ala Gly Gln Arg Ile Asp Cys
260 265 270

Val Val Glu Ala Asn Gln Glu Pro Asp Thr Tyr Trp Ile Asn Ala Pro
275 280 285

Leu Thr Asn Val Pro Asn Lys Thr Ala Gln Ala Leu Leu Val Tyr Glu
290 295 300

Glu Asp Arg Arg Pro Tyr His Pro Pro Lys Gly Pro Tyr Arg Lys Trp
305 310 315 320

Ser Val Ser Glu Ala Ile Ile Lys Tyr Trp Asn His Lys His Lys His
325 330 335

Gly Arg Gly Leu Leu Ser Gly His Gly Gly Leu Lys Ala Arg Met Ile
340 345 350

Glu Gly Ser His His Leu His Ser Arg Ser Val Val Lys Arg Gln Asn
355 360 365

Glu Thr Thr Thr Val Val Met Asp Glu Ser Lys Leu Val Pro Leu Glu
370 375 380

Tyr Pro Gly Ala Ala Cys Gly Ser Lys Pro Ala Asp Leu Val Leu Asp
385 390 395 400

Leu Thr Phe Gly Leu Asn Phe Ala Thr Gly His Trp Met Ile Asn Gly
405 410 415

Ile Pro Tyr Glu Ser Pro Lys Ile Pro Thr Leu Leu Lys Ile Leu Thr
420 425 430

Asp Glu Asp Gly Val Thr Glu Ser Asp Phe Thr Lys Glu Glu His Thr
435 440 445

Val Ile Leu Pro Lys Asn Lys Cys Ile Glu Phe Asn Ile Lys Gly Asn
450 455 460

Ser Gly Ile Pro Ile Thr His Pro Val His Leu His Gly His Thr Trp
465 470 475 480

Asp Val Val Gln Phe Gly Asn Asn Pro Pro Asn Tyr Val Asn Pro Pro
485 490 495

Arg Arg Asp Val Val Gly Ser Thr Asp Ala Gly Val Arg Ile Gln Phe
500 505 510

Lys Thr Asp Asn Pro Gly Pro Trp Phe Leu His Cys His Ile Asp Trp
515 520 525

His Leu Glu Glu Gly Phe Ala Met Val Phe Ala Glu Ala Pro Glu Ala

```
    530                 535                 540

Val Lys Gly Gly Pro Lys Ser Val Ala Val Asp Ser Gln Trp Glu Gly
545                 550                 555                 560

Leu Cys Gly Lys Tyr Asp Asn Trp Leu Lys Ser Asn Pro Gly Gln Leu
                565                 570                 575
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3117 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Rhizoctonia laccase

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: join(393..524, 577..687, 737..799, 860..985, 1043 ..1045, 1097..1219, 1269..1538, 1601..1996, 2047 ..2118, 2174..2284, 2338..2439, 2495..2635, 2693 ..2725, 2786..2899)

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 525..576

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 688..736

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 800..859

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 986..1042

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 1220..1268

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 1539..1600

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 1823..1936

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 1973..2046

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 2119..2173

(ix) FEATURE:
(A) NAME/KEY: intron

```
          (B) LOCATION: 2285..2337

    (ix) FEATURE:
          (A) NAME/KEY: intron
          (B) LOCATION: 2440..2494

    (ix) FEATURE:
          (A) NAME/KEY: intron
          (B) LOCATION: 2636..2692

    (ix) FEATURE:
          (A) NAME/KEY: intron
          (B) LOCATION: 1046..1096


    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GAGTGATCCG CCAGAGTTCA GGCGGATAAG TTCCTAAATA GTCATTCGCC TATTCGTGTA      60

CCTCAGCATA CTGACGACAT ACCGCCAGAT CGCCCTCGGT TCGGGCGTGG CATACGTTCG     120

CAAGGGCACC TCACGGAGCA AACTCTAAAA AGCTTCGGCA TGGATTGCAT TTTGTATTGT     180

AAACAAGTTA CGAGAAAAAC AATAGATCAG TTTTTGCCGA ATCGGATGGC TTGAAACGGA     240

AGTACCGATG GCCGATCCGA GTCGAATGAA TTAACGCATC TGAAACGGGA CCCTGAGTCG     300

AGGCACCCGC CGGCCTTGGC CGTATAAGTC ACTTGTCGCC AACTAGCACT TTTTCATTCC     360

CCCTTTTCTT CTTCCTCGTC TTCTTCTTCT CT ATG GCT CGG TCG ACT ACT TCA       413
                                    Met Ala Arg Ser Thr Thr Ser
                                     1               5

CTC TTT GCA CTG TCT CTC GTT GCT TCA GCG TTT GCT CGA GTC GTT GAC       461
Leu Phe Ala Leu Ser Leu Val Ala Ser Ala Phe Ala Arg Val Val Asp
         10                  15                  20

TAT GGG TTT GAT GTG GCT AAT GGG GCA GTT GCT CCG GAT GGT GTA ACA       509
Tyr Gly Phe Asp Val Ala Asn Gly Ala Val Ala Pro Asp Gly Val Thr
     25                  30                  35

AGG AAC GCG GTT CTC GTGAGTTAGC TGTAAGATGG TGTATATGCT GGTTGCCTAA       564
Arg Asn Ala Val Leu
 40

CGGGAATGTC AG GTC AAT GGT CGC TTC CCT GGT CCA TTG ATC ACC GCC         612
              Val Asn Gly Arg Phe Pro Gly Pro Leu Ile Thr Ala
               45                  50                  55

AAC AAG GGG GAT ACA CTT AAA ATC ACC GTG CGG AAT AAA CTC TCC GAT       660
Asn Lys Gly Asp Thr Leu Lys Ile Thr Val Arg Asn Lys Leu Ser Asp
             60                  65                  70

CCA ACT ATG CGA AGG AGC ACG ACC ATC GTTAGTACTT CCCCTCATCT             707
Pro Thr Met Arg Arg Ser Thr Thr Ile
         75                  80

GTCTTGAAAC TTTCTCATCT TTTTTGAAG CAC TGG CAC GGT CTG CTC CAA CAC       760
                                His Trp His Gly Leu Leu Gln His
                                             85

AGG ACG GCA GAA GAA GAT GGC CCG GCC TTT GTA ACC CAG GTATGCCTTA        809
Arg Thr Ala Glu Glu Asp Gly Pro Ala Phe Val Thr Gln
 90                  95                 100

TCCTATCGCT GCTCTGTCCC CGCGTCCTTC CCTGACTCGG GCGATTCTAG TGC CCG        865
                                                       Cys Pro
```

```
ATT CCT CCG CAA GAA TCG TAC ACC TAT ACG ATG CCG CTC GGC GAA CAG        913
Ile Pro Pro Gln Glu Ser Tyr Thr Tyr Thr Met Pro Leu Gly Glu Gln
105                 110                 115                 120

ACC GGC ACG TAT TGG TAC CAC AGC CAC TTG AGC TCC CAG TAT GTG GAC        961
Thr Gly Thr Tyr Trp Tyr His Ser His Leu Ser Ser Gln Tyr Val Asp
                125                 130                 135

GGG TTG CGT GGG CCC ATC GTT ATT GTAAGTCTTC ATTTAACCTT ATTCTTGGTT      1015
Gly Leu Arg Gly Pro Ile Val Ile
            140

ATGGCTGATT GTGACGTCGT GGTTAGT ATG TTCGTGGCTT CCACAAGAAG               1065
                              Met
                              145

TCAGCAGCCC TTGAAGCTAA CTTTATTCCA G GAC CCC CAC GAC CCG TAC AGA        1117
                                   Asp Pro His Asp Pro Tyr Arg
                                                   150

AAC TAC TAT GAT GTC GAC GAC GAG CGT ACG GTC TTT ACT TTA GCA GAC       1165
Asn Tyr Tyr Asp Val Asp Asp Glu Arg Thr Val Phe Thr Leu Ala Asp
        155                 160                 165

TGG TAC CAC ACG CCG TCG GAG GCT ATC ATT GCC ACC CAC GAT GTC TTG       1213
Trp Tyr His Thr Pro Ser Glu Ala Ile Ile Ala Thr His Asp Val Leu
    170                 175                 180

AAA ACG GTACGCGTTA ATCCTTCTAG CTTTCTTTCC TTGGGTCACT TTCTATCAG         1268
Lys Thr
185

ATC CCC GAC TCG GGT ACG ATC AAC GGC AAA GGC AAA TAC GAT CCT GCT       1316
Ile Pro Asp Ser Gly Thr Ile Asn Gly Lys Gly Lys Tyr Asp Pro Ala
            190                 195                 200

TCG GCT AAC ACC AAC AAC ACG ACA CTC GAG AAC CTC TAC ACT CTC AAA       1364
Ser Ala Asn Thr Asn Asn Thr Thr Leu Glu Asn Leu Tyr Thr Leu Lys
        205                 210                 215

GTC AAA CGC GGC AAG CGG TAT CGC CTG AGG ATT ATC AAC GCC TCG GCC       1412
Val Lys Arg Gly Lys Arg Tyr Arg Leu Arg Ile Ile Asn Ala Ser Ala
    220                 225                 230

ATC GCT TCG TTC CGG TTC GGC GTG CAG GGC CAC AAG TGC ACG ATC ATC       1460
Ile Ala Ser Phe Arg Phe Gly Val Gln Gly His Lys Cys Thr Ile Ile
235                 240                 245                 250

GAG GCT GAT GGC GTC CTC ACC AAA CCG ATC GAG GTC GAT GCG TTT GAT       1508
Glu Ala Asp Gly Val Leu Thr Lys Pro Ile Glu Val Asp Ala Phe Asp
                255                 260                 265

ATT CTA GCA GGC CAG AGG TAT AGC TGC ATC GTAAGTCTAC CTATGCCTTG         1558
Ile Leu Ala Gly Gln Arg Tyr Ser Cys Ile
            270                 275

TTGTGGAGAT AAGAACCTGA CTGAATGTAT GCGCTCCAAT AG TTG AAG GCC GAC        1612
                                               Leu Lys Ala Asp
                                                           280

CAA GAT CCT GAT TCC TAC TGG ATA AAT GCG CCA ATC ACA AAC GTT CTC       1660
Gln Asp Pro Asp Ser Tyr Trp Ile Asn Ala Pro Ile Thr Asn Val Leu
                285                 290                 295

AAC ACC AAC GTC CAG GCA TTG CTA GTG TAT GAA GAT GAC AAG CGT CCT       1708
```

```
Asn Thr Asn Val Gln Ala Leu Leu Val Tyr Glu Asp Asp Lys Arg Pro
            300                 305                 310

ACT CAC TAC CCC TGG AAG CCG TTT TTG ACA TGG AAG ATA TCA AAT GAA        1756
Thr His Tyr Pro Trp Lys Pro Phe Leu Thr Trp Lys Ile Ser Asn Glu
        315                 320                 325

ATC ATT CAG TAC TGG CAG CAC AAG CAC GGG TCG CAC GGT CAC AAG GGA        1804
Ile Ile Gln Tyr Trp Gln His Lys His Gly Ser His Gly His Lys Gly
    330                 335                 340

AAG GGG CAT CAT CAT AAA GTC CGG GCC ATT GGA GGT GTA TCC GGG TTG        1852
Lys Gly His His His Lys Val Arg Ala Ile Gly Gly Val Ser Gly Leu
345                 350                 355                 360

AGC TCC AGG GTT AAG AGC CGG GCG AGT GAC CTA TCG AAG AAG GCT GTC        1900
Ser Ser Arg Val Lys Ser Arg Ala Ser Asp Leu Ser Lys Lys Ala Val
                365                 370                 375

GAG TTG GCT GCT GCA CTC GTT GCG GGT GAG GCC GAG TTG GAC AAG AGG        1948
Glu Leu Ala Ala Ala Leu Val Ala Gly Glu Ala Glu Leu Asp Lys Arg
            380                 385                 390

CAG AAT GAG GAT AAT TCG ACT ATT GTA TTG GAT GAG ACC AAG CTT ATT        1996
Gln Asn Glu Asp Asn Ser Thr Ile Val Leu Asp Glu Thr Lys Leu Ile
        395                 400                 405

GTAAGTCCCT TAATTTTTTT CGGTGTCACG GAAGCTAACC CGCGTAATAG CCG TTG         2052
                                                      Pro Leu
                                                          410

GTT CAA CCT GGT GCA CCG GGC GGC TCC AGA CCA GCT GAC GTC GTG GTC        2100
Val Gln Pro Gly Ala Pro Gly Gly Ser Arg Pro Ala Asp Val Val Val
                415                 420                 425

CCT CTG GAC TTT GGC CTC GTATGTGGCT TCTTGTTATT CGTCCGGAAT               2148
Pro Leu Asp Phe Gly Leu
            430

GCAAACTGAT TTGGGTGGGC TATAG AAC TTT GCC AAC GGA CTG TGG ACG ATA        2200
                            Asn Phe Ala Asn Gly Leu Trp Thr Ile
                                    435                 440

AAC AAT GTC TCC TAC TCC CCT CCG GAT GTC CCT ACT CTC CTC AAG ATC        2248
Asn Asn Val Ser Tyr Ser Pro Pro Asp Val Pro Thr Leu Leu Lys Ile
            445                 450                 455

TTG ACC GAC AAA GAC AAA GTC GAC GCT TCT GAC TTC GTAGGTTCCT             2294
Leu Thr Asp Lys Asp Lys Val Asp Ala Ser Asp Phe
        460                 465

CTTCTTCTTT TCAAACTAGC TACTGACATT AAGTGAACGT CAG ACG GCC GAT GAA        2349
                                                Thr Ala Asp Glu
                                                470

CAC ACG TAT ATT CTT CCA AAG AAC CAA GTT GTC GAG TTG CAC ATC AAG        2397
His Thr Tyr Ile Leu Pro Lys Asn Gln Val Val Glu Leu His Ile Lys
    475                 480                 485

GGA CAG GCT TTG GGA ATC GTA CAC CCC CTT CAT CTG CAT GGC                2439
Gly Gln Ala Leu Gly Ile Val His Pro Leu His Leu His Gly
490                 495                 500

GTACGTCTTT CTCACACTGT TCCAGCTCCT ATTCTCTAAC ACACTCCTGC GATAG CAT       2497
                                                             His
```

GCG TTC GAC GTC GTC CAA TTC GGC GAC AAC GCT CCA AAC TAC GTG AAC 2545
Ala Phe Asp Val Val Gln Phe Gly Asp Asn Ala Pro Asn Tyr Val Asn
505 510 515 520

CCT CCG CGT AGG GAT GTA GTA GGC GTA ACT GAT GCT GGA GTC CGT ATC 2593
Pro Pro Arg Arg Asp Val Val Gly Val Thr Asp Ala Gly Val Arg Ile
525 530 535

CAG TTC AGA ACC GAT AAC CCG GGC CCT TGG TTC CTC CAT TGC 2635
Gln Phe Arg Thr Asp Asn Pro Gly Pro Trp Phe Leu His Cys
540 545 550

GTATGCTCTT CATCTCCCAC CGCTTGTTCT TTACTTATGG TTTACCTTGC GATTTAG 2692

CAC ATT GAT TGG CAC TTG GAA GAA GGA TTT GCT GTAAGTTATT ATTCCTATTC 2745
His Ile Asp Trp His Leu Glu Glu Gly Phe Ala
555 560

CGAAGCATCG GGAGATGCTT AACCAAGGGT GTGTTTTAAG ATG GTA TTC GCC GAA 2800
Met Val Phe Ala Glu
565

GCG CCT GAA GAT ATC AAG AAA GGC TCT CAG AGT GTC AAG CCT GAC GGA 2848
Ala Pro Glu Asp Ile Lys Lys Gly Ser Gln Ser Val Lys Pro Asp Gly
570 575 580

CAA TGG AAG AAA CTA TGC GAG AAG TAT GAG AAG TTG CCT GAA GCA CTG 2896
Gln Trp Lys Lys Leu Cys Glu Lys Tyr Glu Lys Leu Pro Glu Ala Leu
585 590 595

CAG TGAAGTTGCA GTTGTTTCCC ATTCGGGAAC TGGCTCACTA TTCCTTTTGC 2949
Gln

ATAATTCGGA CTTTTATTTT GGGACATTAT TGGACTATGG ACTTGTTTGT CACACCCTCG 3009

CTCACTGTGT CCCTCGTTGA GTACCTATAC TCTATTCGTA TAGTGGGAAT ATGGAATATC 3069

GGATGTAATA AATGCTCGTG CGTTTGGTGC TCGAAATGGG GTAGGACT 3117

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 599 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Ala Arg Ser Thr Thr Ser Leu Phe Ala Leu Ser Leu Val Ala Ser
1 5 10 15

Ala Phe Ala Arg Val Val Asp Tyr Gly Phe Asp Val Ala Asn Gly Ala
20 25 30

Val Ala Pro Asp Gly Val Thr Arg Asn Ala Val Leu Val Asn Gly Arg
35 40 45

Phe Pro Gly Pro Leu Ile Thr Ala Asn Lys Gly Asp Thr Leu Lys Ile
50 55 60

Thr Val Arg Asn Lys Leu Ser Asp Pro Thr Met Arg Arg Ser Thr Thr
65 70 75 80

Ile His Trp His Gly Leu Leu Gln His Arg Thr Ala Glu Glu Asp Gly
85 90 95

Pro Ala Phe Val Thr Gln Cys Pro Ile Pro Pro Gln Glu Ser Tyr Thr
100 105 110

Tyr Thr Met Pro Leu Gly Glu Gln Thr Gly Thr Tyr Trp Tyr His Ser
115 120 125

His Leu Ser Ser Gln Tyr Val Asp Gly Leu Arg Gly Pro Ile Val Ile
130 135 140

Met Asp Pro His Asp Pro Tyr Arg Asn Tyr Tyr Asp Val Asp Asp Glu
145 150 155 160

Arg Thr Val Phe Thr Leu Ala Asp Trp Tyr His Thr Pro Ser Glu Ala
165 170 175

Ile Ile Ala Thr His Asp Val Leu Lys Thr Ile Pro Asp Ser Gly Thr
180 185 190

Ile Asn Gly Lys Gly Lys Tyr Asp Pro Ala Ser Ala Asn Thr Asn Asn
195 200 205

Thr Thr Leu Glu Asn Leu Tyr Thr Leu Lys Val Lys Arg Gly Lys Arg
210 215 220

Tyr Arg Leu Arg Ile Ile Asn Ala Ser Ala Ile Ala Ser Phe Arg Phe
225 230 235 240

Gly Val Gln Gly His Lys Cys Thr Ile Ile Glu Ala Asp Gly Val Leu
245 250 255

Thr Lys Pro Ile Glu Val Asp Ala Phe Asp Ile Leu Ala Gly Gln Arg
260 265 270

Tyr Ser Cys Ile Leu Lys Ala Asp Gln Asp Pro Asp Ser Tyr Trp Ile
275 280 285

Asn Ala Pro Ile Thr Asn Val Leu Asn Thr Asn Val Gln Ala Leu Leu
290 295 300

Val Tyr Glu Asp Asp Lys Arg Pro Thr His Tyr Pro Trp Lys Pro Phe
305 310 315 320

Leu Thr Trp Lys Ile Ser Asn Glu Ile Ile Gln Tyr Trp Gln His Lys
325 330 335

His Gly Ser His Gly His Lys Gly Lys Gly His His His Lys Val Arg
340 345 350

Ala Ile Gly Gly Val Ser Gly Leu Ser Ser Arg Val Lys Ser Arg Ala
355 360 365

Ser Asp Leu Ser Lys Lys Ala Val Glu Leu Ala Ala Ala Leu Val Ala
370 375 380

Gly Glu Ala Glu Leu Asp Lys Arg Gln Asn Glu Asp Asn Ser Thr Ile
385 390 395 400

Val Leu Asp Glu Thr Lys Leu Ile Pro Leu Val Gln Pro Gly Ala Pro
405 410 415

Gly Gly Ser Arg Pro Ala Asp Val Val Val Pro Leu Asp Phe Gly Leu
420 425 430

Asn Phe Ala Asn Gly Leu Trp Thr Ile Asn Asn Val Ser Tyr Ser Pro

```
        435                     440                     445

Pro Asp Val Pro Thr Leu Leu Lys Ile Leu Thr Asp Lys Asp Lys Val
    450                     455                     460

Asp Ala Ser Asp Phe Thr Ala Asp Glu His Thr Tyr Ile Leu Pro Lys
465                     470                     475                     480

Asn Gln Val Val Glu Leu His Ile Lys Gly Gln Ala Leu Gly Ile Val
                485                     490                     495

His Pro Leu His Leu His Gly His Ala Phe Asp Val Val Gln Phe Gly
            500                     505                     510

Asp Asn Ala Pro Asn Tyr Val Asn Pro Pro Arg Arg Asp Val Val Gly
        515                     520                     525

Val Thr Asp Ala Gly Val Arg Ile Gln Phe Arg Thr Asp Asn Pro Gly
    530                     535                     540

Pro Trp Phe Leu His Cys His Ile Asp Trp His Leu Glu Glu Gly Phe
545                     550                     555                     560

Ala Met Val Phe Ala Glu Ala Pro Glu Asp Ile Lys Lys Gly Ser Gln
                565                     570                     575

Ser Val Lys Pro Asp Gly Gln Trp Lys Lys Leu Cys Glu Lys Tyr Glu
            580                     585                     590

Lys Leu Pro Glu Ala Leu Gln
        595
```

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
Ala Val Arg Asn Tyr Lys Phe Asp Ile Lys Asn Val Asn Val Ala Pro
1               5                   10                  15

Asp Gly Phe Gln Arg Pro Ile Val Ser Val
            20                  25
```

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```
Ser Gln Tyr Val Asp Gly Leu Arg Gly Pro Leu Val Ile Tyr Asp Pro
1               5                   10                  15

Asp Asp Asp His
            20
```

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 amino acids

(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```
Ser Arg Tyr Asx Val Asx Asx Ala Ser Thr Val Val Met Leu Glu Asx
1               5                   10                  15

Trp Tyr Arg Thr Pro Ala Xaa Val Leu Glu
            20                  25
```

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

```
Ser Leu Gly Pro Thr Pro Asn Tyr Val Asn Pro Xaa Ile Arg Asp Val
1               5                   10                  15

Val Arg Val Gly Gly Thr Thr Val Val
            20                  25
```

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

```
Gly Leu Ala Leu Val Phe Ala Glu Ala Pro Ser Gln Ile Arg Gln Gly
1               5                   10                  15

Val Gln Ser Val Gln Pro Asp Asp Ala
            20                  25
```

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

```
Ile Arg Tyr Val Gly Gly Pro Ala Val Xaa Arg Ser Val Ile
1               5                   10
```

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 6 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

```
Ile Leu Ala Asn Pro Ala
1               5
```

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

```
Tyr Glu Ala Pro Ser Leu Pro Thr
1               5
```

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1912 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Rhizoctonia laccase

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 85..1671

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

```
CTAACGCTTG GTGCCGAGCT CGGATCCACT AGTAACGCGC GCCAGTGTGC TGGAATTCGC       60

GGCCGCGTCG ACACCTCCTT CAAG ATG CTT TCT AGC ATT ACC CTC CTA CCT         111
                           Met Leu Ser Ser Ile Thr Leu Leu Pro
                            1               5

TTG CTC GCT GCG GTC TCA ACC CCC GCC TTT GCT GCC GTC CGC AAC TAT        159
Leu Leu Ala Ala Val Ser Thr Pro Ala Phe Ala Ala Val Arg Asn Tyr
 10                  15                  20                  25

AAG TTC GAC ATC AAG AAC GTC AAT GTC GCT CCC GAT GGC TTT CAG CGC        207
Lys Phe Asp Ile Lys Asn Val Asn Val Ala Pro Asp Gly Phe Gln Arg
                 30                  35                  40

TCT ATC GTC TCC GTC AAC GGT TTA GTT CCT GGC ACG TTG ATC ACG GCC        255
Ser Ile Val Ser Val Asn Gly Leu Val Pro Gly Thr Leu Ile Thr Ala
             45                  50                  55

AAC AAG GGT GAC ACC TTG CGC ATT AAT GTC ACG AAT CAA CTC ACG GAC        303
Asn Lys Gly Asp Thr Leu Arg Ile Asn Val Thr Asn Gln Leu Thr Asp
         60                  65                  70

CCT AGT ATG CGT CGT GCC ACA ACG ATT CAT TGG CAT GGA TTG TTC CAA        351
Pro Ser Met Arg Arg Ala Thr Thr Ile His Trp His Gly Leu Phe Gln
     75                  80                  85
```

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GCT ACT ACC GCC GAC GAG GAT GGC CCC GCA TTC GTC ACG CAA TGC CCT        399
Ala Thr Thr Ala Asp Glu Asp Gly Pro Ala Phe Val Thr Gln Cys Pro
 90                 95                 100                 105

ATT GCG CAA AAT TTG TCC TAT ACA TAC GAG ATC CCA TTG CGC GGC CAA        447
Ile Ala Gln Asn Leu Ser Tyr Thr Tyr Glu Ile Pro Leu Arg Gly Gln
                110                 115                 120

ACA GGA ACC ATG TGG TAT CAC GCC CAT CTT GCG AGT CAA TAT GTC GAT        495
Thr Gly Thr Met Trp Tyr His Ala His Leu Ala Ser Gln Tyr Val Asp
            125                 130                 135

GGA TTG CGA GGC CCT TTG GTC ATC TAT GAT CCA AAC GAC CCA CAC AAG        543
Gly Leu Arg Gly Pro Leu Val Ile Tyr Asp Pro Asn Asp Pro His Lys
        140                 145                 150

TCG CGC TAC GAC GTG GAT GAT GCG AGC ACA GTA GTC ATG CTT GAG GAC        591
S r Arg Tyr Asp Val Asp Asp Ala Ser Thr Val Val Met Leu Glu Asp
    155                 160                 165

TGG TAC CAT ACT CCG GCA CCC GTT CTA GAA AAG CAA ATG TTC TCG ACT        639
Trp Tyr His Thr Pro Ala Pro Val Leu Glu Lys Gln Met Phe Ser Thr
170                 175                 180                 185

AAT AAC ACC GCT CTG CTC TCT CCT GTT CCG GAC TCG GGT CTT ATC AAT        687
Asn Asn Thr Ala Leu Leu Ser Pro Val Pro Asp Ser Gly Leu Ile Asn
                190                 195                 200

GGC AAA GGG CGC TAT GTG GGC GGT CCC GCA GTT CCC CGG TCA GTA ATC        735
Gly Lys Gly Arg Tyr Val Gly Gly Pro Ala Val Pro Arg Ser Val Ile
            205                 210                 215

AAC GTA AAA CGT GGG AAA CGA TAT CGC TTG CGC GTA ATC AAC GCT TCT        783
Asn Val Lys Arg Gly Lys Arg Tyr Arg Leu Arg Val Ile Asn Ala Ser
        220                 225                 230

GCT ATC GGG TCG TTT ACC TTT TCG ATC GAA GGA CAT AGT CTG ACT GTC        831
Ala Ile Gly Ser Phe Thr Phe Ser Ile Glu Gly His Ser Leu Thr Val
    235                 240                 245

ATT GAG GCC GAT GGG ATC CTG CAC CAG CCC TTG GCT GTT GAC AGC TTC        879
Ile Glu Ala Asp Gly Ile Leu His Gln Pro Leu Ala Val Asp Ser Phe
250                 255                 260                 265

CAG ATT TAC GCT GGA CAA CGC TAC TCT GTC ATC GTT GAA GCC AAC CAA        927
Gln Ile Tyr Ala Gly Gln Arg Tyr Ser Val Ile Val Glu Ala Asn Gln
                270                 275                 280

ACC GCC GCC AAC TAC TGG ATT CGT GCA CCA ATG ACC GTT GCA GGA GCC        975
Thr Ala Ala Asn Tyr Trp Ile Arg Ala Pro Met Thr Val Ala Gly Ala
            285                 290                 295

GGA ACC AAT GCA AAC TTG GAC CCC ACC AAT GTC TTT GCC GTA TTG CAC       1023
Gly Thr Asn Ala Asn Leu Asp Pro Thr Asn Val Phe Ala Val Leu His
        300                 305                 310

TAC GAG GGA GCG CCC AAC GCC GAA CCC ACG ACG GAA CAA GGC AGT GCT       1071
Tyr Glu Gly Ala Pro Asn Ala Glu Pro Thr Thr Glu Gln Gly Ser Ala
    315                 320                 325

ATC GGT ACT GCA CTC GTT GAA GAG AAC CTC CAT GCG CTC ATC AAC CCT       1119
Ile Gly Thr Ala Leu Val Glu Glu Asn Leu His Ala Leu Ile Asn Pro
330                 335                 340                 345

GGC GCT CCG GGC GGC TCC GCT CCC GCA GAC GTT TCC CTC AAT CTT GCA       1167
Gly Ala Pro Gly Gly Ser Ala Pro Ala Asp Val Ser Leu Asn Leu Ala
                350                 355                 360
```

ATT GGG CGC AGC ACA GTT GAT GGG ATT CTT AGG TTC ACA TTT AAT AAC 1215
Ile Gly Arg Ser Thr Val Asp Gly Ile Leu Arg Phe Thr Phe Asn Asn
365 370 375

ATC AAG TAC GAG GCT CCT TCG TTG CCC ACG CTC TTG AAG ATT TTG GCA 1263
Ile Lys Tyr Glu Ala Pro Ser Leu Pro Thr Leu Leu Lys Ile Leu Ala
380 385 390

AAC AAT GCG AGC AAT GAC GCC GAT TTC ACG CCA AAT GAG CAC ACT ATC 1311
Asn Asn Ala Ser Asn Asp Ala Asp Phe Thr Pro Asn Glu His Thr Ile
395 400 405

GTA TTG CCA CAC AAT AAA GTT ATC GAG CTC AAT ATC ACC GGA GGT GCA 1359
Val Leu Pro His Asn Lys Val Ile Glu Leu Asn Ile Thr Gly Gly Ala
410 415 420 425

GAC CAC CCT ATC CAT CTC CAC GGC CAT GTG TTT GAT ATC GTC AAA TCA 1407
Asp His Pro Ile His Leu His Gly His Val Phe Asp Ile Val Lys Ser
430 435 440

CTC GGT GGT ACC CCG AAC TAT GTC AAC CCG CCA CGC AGG GAC GTA GTT 1455
Leu Gly Gly Thr Pro Asn Tyr Val Asn Pro Pro Arg Arg Asp Val Val
445 450 455

CGT GTC GGA GGC ACC GGT GTG GTA CTC CGA TTC AAG ACC GAT AAC CCA 1503
Arg Val Gly Gly Thr Gly Val Val Leu Arg Phe Lys Thr Asp Asn Pro
460 465 470

GGC CCA TGG TTT GTT CAC TGC CAC ATT GAC TGG CAC TTG GAG GCT GGG 1551
Gly Pro Trp Phe Val His Cys His Ile Asp Trp His Leu Glu Ala Gly
475 480 485

CTC GCA CTT GTC TTT GCC GAG GCC CCC AGC CAG ATT CGC CAG GGT GTC 1599
Leu Ala Leu Val Phe Ala Glu Ala Pro Ser Gln Ile Arg Gln Gly Val
490 495 500 505

CAG TCG GTC CAG CCC AAC AAT GCC TGG AAC CAG CTC TGC CCC AAG TAC 1647
Gln Ser Val Gln Pro Asn Asn Ala Trp Asn Gln Leu Cys Pro Lys Tyr
510 515 520

GCG GCT CTT CCT CCC GAT TTG CAG T 1672
Ala Ala Leu Pro Pro Asp Leu Gln
525

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 529 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Met Leu Ser Ser Ile Thr Leu Leu Pro Leu Leu Ala Ala Val Ser Thr
1 5 10 15

Pro Ala Phe Ala Ala Val Arg Asn Tyr Lys Phe Asp Ile Lys Asn Val
20 25 30

Asn Val Ala Pro Asp Gly Phe Gln Arg Ser Ile Val Ser Val Asn Gly
35 40 45

Leu Val Pro Gly Thr Leu Ile Thr Ala Asn Lys Gly Asp Thr Leu Arg
50 55 60

```
Ile Asn Val Thr Asn Gln Leu Thr Asp Pro Ser Met Arg Arg Ala Thr
 65                 70                  75                  80

Thr Ile His Trp His Gly Leu Phe Gln Ala Thr Thr Ala Asp Glu Asp
                85                  90                  95

Gly Pro Ala Phe Val Thr Gln Cys Pro Ile Ala Gln Asn Leu Ser Tyr
            100                 105                 110

Thr Tyr Glu Ile Pro Leu Arg Gly Gln Thr Gly Thr Met Trp Tyr His
        115                 120                 125

Ala His Leu Ala Ser Gln Tyr Val Asp Gly Leu Arg Gly Pro Leu Val
    130                 135                 140

Ile Tyr Asp Pro Asn Asp Pro His Lys Ser Arg Tyr Asp Val Asp Asp
145                 150                 155                 160

Ala Ser Thr Val Val Met Leu Glu Asp Trp Tyr His Thr Pro Ala Pro
                165                 170                 175

Val Leu Glu Lys Gln Met Phe Ser Thr Asn Asn Thr Ala Leu Leu Ser
            180                 185                 190

Pro Val Pro Asp Ser Gly Leu Ile Asn Gly Lys Gly Arg Tyr Val Gly
        195                 200                 205

Gly Pro Ala Val Pro Arg Ser Val Ile Asn Val Lys Arg Gly Lys Arg
    210                 215                 220

Tyr Arg Leu Arg Val Ile Asn Ala Ser Ala Ile Gly Ser Phe Thr Phe
225                 230                 235                 240

Ser Ile Glu Gly His Ser Leu Thr Val Ile Glu Ala Asp Gly Ile Leu
                245                 250                 255

His Gln Pro Leu Ala Val Asp Ser Phe Gln Ile Tyr Ala Gly Gln Arg
            260                 265                 270

Tyr Ser Val Ile Val Glu Ala Asn Gln Thr Ala Ala Asn Tyr Trp Ile
        275                 280                 285

Arg Ala Pro Met Thr Val Ala Gly Ala Gly Thr Asn Ala Asn Leu Asp
    290                 295                 300

Pro Thr Asn Val Phe Ala Val Leu His Tyr Glu Gly Ala Pro Asn Ala
305                 310                 315                 320

Glu Pro Thr Thr Glu Gln Gly Ser Ala Ile Gly Thr Ala Leu Val Glu
                325                 330                 335

Glu Asn Leu His Ala Leu Ile Asn Pro Gly Ala Pro Gly Gly Ser Ala
            340                 345                 350

Pro Ala Asp Val Ser Leu Asn Leu Ala Ile Gly Arg Ser Thr Val Asp
        355                 360                 365

Gly Ile Leu Arg Phe Thr Phe Asn Asn Ile Lys Tyr Glu Ala Pro Ser
    370                 375                 380

Leu Pro Thr Leu Leu Lys Ile Leu Ala Asn Asn Ala Ser Asn Asp Ala
385                 390                 395                 400

Asp Phe Thr Pro Asn Glu His Thr Il  Val Leu Pro His Asn Lys Val
                405                 410                 415

Ile Glu Leu Asn Ile Thr Gly Gly Ala Asp His Pro Ile His Leu His
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                420                     425                     430

Gly His Val Phe Asp Ile Val Lys Ser Leu Gly Gly Thr Pro Asn Tyr
        435                     440                     445

Val Asn Pro Pro Arg Arg Asp Val Val Arg Val Gly Gly Thr Gly Val
    450                     455                     460

Val Leu Arg Phe Lys Thr Asp Asn Pro Gly Pro Trp Phe Val His Cys
465                     470                     475                     480

His Ile Asp Trp His Leu Glu Ala Gly Leu Ala Leu Val Phe Ala Glu
                485                     490                     495

Ala Pro Ser Gln Ile Arg Gln Gly Val Gln Ser Val Gln Pro Asn Asn
            500                     505                     510

Ala Trp Asn Gln Leu Cys Pro Lys Tyr Ala Ala Leu Pro Pro Asp Leu
        515                     520                     525

Gln
```

What we claim is:

1. A nucleic acid fragment containing a nucleic acid sequence encoding a *Rhizoctonia* laccase which functions optimally at pH between about 6.0 and 8.5.

2. The fragment of Claim 1 which comprises a sequence encoding a *Rhizoctonia solani* laccase.

3. The fragment of Claim 1 which comprises a nucleic acid sequence encoding the amino acid sequence depicted in SEQ ID NO. 2.

4. The fragment of Claim 1 which comprises a nucleic acid sequence encoding the amino acid sequence depicted in SEQ ID NO. 4.

5. The fragment of Claim 1, which comprises a nucleic acid sequence encoding a protein containing one or more of the amino acid sequences depicted in SEQ. ID NOS. 5, 6, 7, 8, 9, 10, 11, or 12.

6. The fragment of Claim 1 which comprises a nucleic acid sequence encoding the amino acid sequence depicted in SEQ ID NO. 14.

7. The fragment of Claim 1, which comprises the nucleic acid sequence depicted in SEQ ID NO. 1.

8. The fragment of Claim 1, which comprises the nucleic acid sequence depicted in SEQ. ID. NO. 3.

9. The fragment of Claim 1, which comprises the nucleic acid sequence depicted in SEQ. ID. NO. 13.

10. The fragment of Claim 1, which comprises the nucleic acid sequence contained in NRRL B-21141.

11. The fragment of Claim 1, which comprises the nucleic acid sequence contained in NRRL B-21142.

12. The fragment of Claim 1, which comprises the nucleic acid sequence encoding the laccase produced by RS 22.

13. The fragment of Claim 1, which comprises the nucleic acid sequence contained in NRRL B-21156.

14. A substantially pure *Rhizoctonia* laccase enzyme which functions optimally at a pH between about 6.0-8.5.

15. The enzyme of Claim 14 which is a *Rhizoctonia solani* laccase.

16. The enzyme of Claim 14 which comprises the sequence depicted in SEQ ID NO. 2, or a sequence with at least 80% homology thereto.

17. The enzyme of Claim 14 which comprises the sequence depicted in SEQ ID NO 4, or a sequence with at least 80% homology thereto.

18. The enzyme of Claim 14 which comprises one or more of the peptide sequences depicted in SEQ ID NOS.5, 6, 7,

8, 9, 10, 11 or 12, or a sequence with at least 80% homology to one or more of these peptides.

19. The enzyme of Claim 14 which comprises the sequence depicted in SEQ ID NO 14, or a sequence with at least 80% homology thereto.

20. A recombinant vector comprising a nucleic acid fragment containing a nucleic acid sequence encoding a *Rhizoctonia* laccase which functions optimally at pH between about 6.0-8.5.

21. The vector of Claim 20 in which the fragment is operably linked to a promoter sequence.

22. The vector of Claim 21 in which the promoter is a fungal or yeast promoter.

23. The vector of Claim 22 in which the promoter is the TAKA amylase promoter of *Aspergillus oryzae.*

24. The vector of Claim 22 in which the promoter is the glucoamylase (gluA) promoter of *Aspergillus niger* or *Aspergillus awamsii.*

25. The vector of Claim 21 which also comprises a selectable marker.

26. The vector of Claim 25 in which the selectable marker is the amdS marker of *Aspergillus nidulans* or *Aspergillus oryzae.*

27. The vector of Claim 25 in which the selectable marker is the pyrG marker of *Aspergillus nidulans, Aspergillus niger, Aspergillus awamorii*, or *Aspergillus oryzae*.

28. The vector of Claim 21 which comprises both the TAKA amylase promoter of *Aspergillus oryzae* and the amdS or pyrG marker of *Aspergillus nidulans* or *Aspergillus oryzae*.

29. A host cell comprising a heterologous nucleic acid fragment containing a nucleic acid sequence encoding a *Rhizoctonia* laccase which functions optimally at pH between about 6.0-8.5.

30. The host cell of Claim 28 which is a fungal cell.

31. The host cell of Claim 30 which is an *Aspergillus* cell.

32. The host cell of Claim 29 in which the fragment is integrated into the host cell genome.

33. The host cell of Claim 29 in which the fragment is contained on a vector.

34. The host cell of Claim 29 which comprises a fragment containing a sequence encoding the amino acid sequence depicted in SEQ ID NO. 2.

35. The host cell of Claim 29 which comprises a fragment containing a sequence encoding the amino acid sequence depicted in SEQ ID NO: 4.

36. The host cell of Claim 29 which comprises a fragment containing a sequence encoding the amino acid sequence depicted in SEQ ID NO: 14.

37. The host cell of Claim 29 which comprises a fragment containing a sequence encoding one or more of the amino acid sequences depicted in SEQ ID NOS.: 5, 6, 7, 8, 9, 10, 11, or 12.

38. A method for obtaining a laccase enzyme which functions optimally at a pH between about 6.0-8.5 which comprises culturing a host cell comprising a nucleic acid fragment containing a nucleic acid sequence encoding a *Rhizoctonia* laccase enzyme which functions optimally at a pH between about 6.0-8.5, under conditions conducive to expression of the enzyme, and recovering the enzyme from the culture.

39. A method for polymerizing a lignin or lignosulfate substrate in solution which comprises contacting the substrate with a *Rhizoctonia* laccase which functions optimally at a pH between about 6.0-8.5.

40. A method for in situ depolymerization in Kraft pulp which comprises contacting the pulp with a *Rhizoctonia* laccase which functions optimally at a pH between about 6.0-8.5.

41. A method for oxidizing dyes which comprises contacting the dye with a *Rhizoctonia* laccase which functions optimally at a pH between about 6.0-8.5.

42. A method of polymerizing a phenolic compounds which comprises contacting the phenolic compound with a *Rhizoctonia* laccase which functions optimally at a pH between about 6.0-8.5.

```
  1 AGCGTCACACCAGACATCGGATGAAAACGGAAAGTGTATGCGCCATTTGACGTCTGCGGC  60

 61 AACCACTGTTCATCTCGCGAGCTAACATGGGCGACGTATAAGAAGAACGCGAGAATGGGC 120

121 AGATTTCGATATCCCCTCTCGTCTCGGTTTTGGTCTCGGCTTGCCTCTAATGGCGCGCAC 180
                                                    M  A  R  T    4

181 CACTTTCCTTGTCTCGGTTTCGCTCTTTGTTTCCGCTGTTCTTGCGCGCACCGTCGAGTA 240
  4  T  F  L  V  S  V  S  L  F  V  S  A  V  L  A  R  T  V  E  Y   24

241 CGGCTTGAAGATTAGTGATGGGGAGATAGCTCCTGACGGTGTTAAGCGTAATGCGACTTT 300
 24  G  L  K  I  S  D  G  E  I  A  P  D  G  V  K  R  N  A  T  L   44

301 GGgtacgcactccttgtaatccaacaattcaaggtttctgatgcttggtcagTAAATGGA 360
 44                                                     V  N  G   47

361 GGGTATCCCGGTCCACTCATTTTTGCCAACAAGGGGGATACTCTCAAAGTCAAGGTCCAA 420
 47 G  Y  P  G  P  L  I  F  A  N  K  G  D  T  L  K  V  K  V  Q    67

421 AACAAGCTCACGAATCCTGAGATGTATCGCACCACTTCCATCgtatgttcgttcgatatc 480
 67 N  K  L  T  N  P  E  M  Y  R  T  T  S  I                      81

481 tactaatacatccgtcgctaaatatcttgtagCATTGGCACGGTCTCTTACAACATAGAA 540
 81                                 H  W  H  G  L  L  Q  H  R      90
```

FIG. 1A

```
541 ACGCCGACGACGACGGTCCTTCGTTCGTCACTCAGgtaggattctggaaggttggcctga  600
 90 N  A  D  D  D  G  P  S  F  V  T  Q                              102

601 actctctgttaaccgacaacccgatgtcaccagTGCCCGATTGTTCCACGCGAGTCGTAT  660
102                                  C  P  I  V  P  R  E  S  Y      111

661 ACTTACACCATACCTCTGGACGATCAAACCGGAACCTATTGGTACCATAGCCACTTGAGT  720
111  T  Y  T  I  P  L  D  D  Q  T  G  T  Y  W  Y  H  S  H  L  S    131

721 TCGCAATACGTTGATGGTCTTCGAGGCCCGCTGGTAATCTgtgagtatcttgacttgtct  780
131  S  Q  Y  V  D  G  L  R  G  P  L  V  I                          144

781 actgaaggcaacgagactaaaacaagcgtcgattcacagATGgttcgtctcccctttatt  840
144                                        Y                        145

841 tagctctggatcttcatttctcacgtaatacatgatagATCCCAAGGATCCTCACAGGCG  900
144                                       D  P  K  D  P  H  R  R    152

901 TTTGTATGATGTTGACGATGAGAAGACCGTCCTGATCATCGGTGACTGGTATCATGAATC  960
152  L  Y  D  V  D  D  E  K  T  V  L  I  I  G  D  W  Y  H  E  S    172

961 GTCCAAGGCAATCCTTGCTTCTGGTAACATTACCCGACAgtaagtgatacatgccggtcc 1020
172  S  K  A  I  L  A  S  G  N  I  T  R  Q                          185
```

FIG. 1B

```
1021 cagaaaattctctaaattcattttaattacagGCGACCGGTCTCTGCCACCATCAACGG 1080
185                                   R  P  V  S  A  T  I  N  G  194

1081 CAAAGGTCGATTTGACCCTGACAACACTCCTGCCAACCCAGATACTCTGTACACCCTCAA 1140
194   K  G  R  F  D  P  D  N  T  P  A  N  P  D  T  L  Y  T  L  K  214

1141 GGTCAAGCGAGGGAAGCGCTATCGTCTGCGTGTCATCAATAGCTCGGAGATCGCTTCGTT 1200
214   V  K  R  G  K  R  Y  R  L  R  V  I  N  S  S  E  I  A  S  F  234

1201 CCGATTCAGTGTGGAAGGTCACAAGGTGACTGTGATTGCTGCCGATGGCGTCTCTACCAA 1260
234   R  F  S  V  E  G  H  K  V  T  V  I  A  A  D  G  V  S  T  K  254

1261 ACCGTATCAGGTCGATGCGTTTGATATTCTAGCAGGACAGCGCATAGATTGCGTCgtaag 1320
254   P  Y  Q  V  D  A  F  D  I  L  A  G  Q  R  I  D  C  V     272

1321 tgtcgtccgaacccacatctgagctcaagtgttgatacatgcgcgcttatagGTGGAGGC 1380
272                                                     V  E  A  275

1381 GAACCAAGAACCCGACACATACTGGATCAACGCACCGCTGACCAACGTGCCCAACAAGAC 1440
275   N  Q  E  P  D  T  Y  W  I  N  A  P  L  T  N  V  P  N  K  T  295

1441 CGCTCAGGCTCTCCTCGTTTATGAGGAGGATCGTCGGCCGTACCACCCTCCAAAGGGCCC 1500
295   A  Q  A  L  L  V  Y  E  E  D  R  R  P  Y  H  P  P  K  G  P  315

1501 GTATCGCAAGTGGAGCGTCTCTGAGGCGATCATCAAGTACTGGAATCACAAGCACAAGCA 1560
315   Y  R  K  W  S  V  S  E  A  I  I  K  Y  W  N  H  K  H  K  H  335
```

FIG. 1C

```
1561 CGGACGTGGTTTGCTGTCTGGACATGGAGGTCTCAAGGCTCGGATGATCGAGGGTAGCCA 1620
335   G  R  G  L  L  S  G  H  G  G  L  K  A  R  M  I  E  G  S  H   340

1621 TCATCTGCATTCGCGCAGCGTCGTTAAGCGCCAGAATGAGACCACCACTGTTGTAATGGA 1680
340   H  L  H  S  R  S  V  V  K  R  Q  N  E  T  T  T  V  V  M  D   350

1681 CGAGAGCAAGCTCGTTgtaagtaccatatttaaaagttggttgggtttcgaatacttatt 1740
350   E  S  K  L  V

1741 tcaacttttcttagCCACTGGAATACCCCGGCGCTGCATGCGGGTCTAAACCTGCTGACC 1800
350                P  L  E  Y  P  G  A  A  C  G  S  K  P  A  D   365

1801 TCGTCTTGGATCTCACTTTTGGTTTGgtatgtagccaaatcgcccatatacaggatactg 1860
365  L  V  L  D  L  T  F  G  L                                    374

1861 aatattgtttgtgcgtgtagAACTTTGCTACCGGGCACTGGATGATCAACGGTATCCCAT 1920
374                      N  F  A  T  G  H  W  M  I  N  G  I  P     387

1921 ACGAGTCTCCCAAAATCCCCACATTGCTCAAGATCCTCACTGATGAGGACGGGGTTACCG 1980
387  Y  E  S  P  K  I  P  T  L  L  K  I  L  T  D  E  D  G  V  T    407

1981 AGTCTGACTTgtatgttcccttttcggtatcttcgtatgcgtgcactgactcgtgctggt 2040
407  E  S  D  F                                                   411

2041 gggaatttagCACCAAGGAGGAGCACACAGTCATACTCCCGAAGAACAAATGCATCGAAT 2100
411            T  K  E  E  H  T  V  I  L  P  K  N  K  C  I  E      427
```

FIG. 1D

```
2101 TCAACATCAAGGGGAACTCGGGTATTCCCATTACGCACCCCGTACATCTTCACGGTgtaa 2160
427  F  N  I  K  G  N  S  G  I  P  I  T  H  P  V  H  L  H  G      446

2161 gtgcatatcggatggtttacgatactaaggctcatcaacttttttagCACACTTGGGATGT 2220
446                                                 H  T  W  D  V  451

2221 CGTACAATTTGGCAACAACCCACCCAATTATGTCAATCCTCCCCGTAGGGACGTGGTTGG 2280
451   V  Q  F  G  N  N  P  P  N  Y  V  N  P  P  R  R  D  V  V  G  471

2281 CTCTACAGATGCGGGTGTGAGGATTCAGTTCAAGACCGACAATCCAGGACCGTGGTTCCT 2340
471   S  T  D  A  G  V  R  I  Q  F  K  T  D  N  P  G  P  W  F  L  491

2341 GCACTGgtgcgtcggtccccatcgtccgttatggtttttctaatacgtcccattctattt 2400
491   H  C                                                          493

2401 tagCCATATTGACTGGCATCTTGAGGAGGGTTTCGCAAgtgagtactgagacctaagtgc 2460
493     H  I  D  W  H  L  E  E  G  F  A                             504

2461 tactcggctcattactgattaccgcatgtatgcgtctagTGGTGTTTGCTGAAGCGCCCG 2520
504                                            M  V  F  A  E  A  P  511

2521 AAGCCGTCAAGGGAGGTCCAAAGAGCGTGGCCGTGGACTCTCAGTGGGAAGGGCTGTGTG 2580
511  E  A  V  K  G  G  P  K  S  V  A  V  D  S  Q  W  E  G  L  C     531

2581 GCAAGTACGACAACTGGCTAAAATCAAATCCGGGCCAGCTGTAGGCGTATCGCAGCCACA 2640
531  G  K  Y  D  N  W  L  K  S  N  P  G  Q  L  *                   545
```

FIG. 1E

2641 TTGGTGATGATTGAAAGTTGCATCTTGTTCCTATAACCGGCTCTTATATACGGGTGTCTC 2700

2701 CCAGTAAAGTCGTAGCCCAATTTCAGCCGAGACAGATATTTAGTGGACTCTTACTCTTGT 2760

2761 GTCCCATTGACGCACATCGTTGCATCAAACCTGCTTTTTATCGTCCCTCTTTGTAATTTG 2820

2821 TGTTGCTGTAATGTATCG 2838

F I G. 1F

```
  1 AAGCTTCGGCATGGATTGCATTTTGTATTGT                                180

181 AAACAAGTTACGAGAAAAACAATAGATCAGTTTTTGCCGAATCGGATGGCTTGAAACGGA 240

241 AGTACCGATGGCCGATCCGAGTCGAATGAATTAACGCATCTGAAACGGGACCCTGAGTCG 300

301 AGGCACCCGCCGGCCTTGGCCGTATAAGTCACTTGTCGCCAACTAGCACTTTTTCATTCC 360

361 CCCTTTTCTTCTTCCTCGTCTTCTTCTTCTCTATGGCTCGGTCGACTACTTCACTCTTTG 420
  1                                  M  A  R  S  T  T  S  L  F   10

421 CACTGTCTCTGGCCGCACCGGCCTTGGCTCGAGTCGTTGACTATGGGTTTGATGTGGCTA 480
 10 A  L  S  L  A  A  P  A  L  A  R  V  V  D  Y  G  F  D  V  A   30

481 ATGGGGCAGTTGCTCCGGATGGTGTAACAAGGAACGCGGTTCTCGgtgagttagctgtaa 540
 30 N  G  A  V  A  P  D  G  V  T  R  N  A  V  L                  45

541 gatggtgtatatgctggttgcctaacgggaatgtcagTCAATGGTCGCTTCCCTGGTCCA 600
 45                                      V  N  G  R  F  P  G  P  53

601 TTGATCACCGCCAACAAGGGGGATACACTTAAAATCACCGTGCGGAATAAACTCTCCGAT 660
 53  L  I  T  A  N  K  G  D  T  L  K  I  T  V  R  N  K  L  S  D  73
```

FIG. 2A

```
661  CCAACTATGCGAAGGAGCACGACCATCgttagtacttcccctcatctgtcttgaaacttt  720
73    P  T  M  R  R  S  T  T  I                                     82

721  ctcatctttttgaagCACTGGCACGGTCTGCTCCAACACAGGACGGCAGAAGAAGATGG  780
82                   H  W  H  G  L  L  Q  H  R  T  A  E  E  D  G    97

781  CCCGGCCTTTGTAACCCAGgtatgccttatcctatcgctgctctgtccccgcgtccttcc  840
97    P  A  F  V  T  Q                                              103

841  ctgactcgggcgattctagTGCCCGATTCCTCCGCAAGAATCGTACACCTATACGATGCC  900
103                     C  P  I  P  P  Q  E  S  Y  T  Y  T  M  P    117

901  GCTCGGCGAACAGACCGGCACGTATTGGTACCACAGCCACTTGAGCTCCCAGTATGTGGA  960
117   L  G  E  Q  T  G  T  Y  W  Y  H  S  H  L  S  S  Q  Y  V  D    137

961  CGGGTTGCGTGGGCCCATCGTTATTTgtaagtcttcatttaaccttattcttggctatgg 1020
137   G  L  R  G  P  I  V  I                                        145

1021 ctgattgtgacgtcgtggttagATGgttcgtggcttccacaagaagtcagcagcccttga 1080
145                          Y                                      145

1081 agctaactttattccagACCCCCACGACCCGTACAGAAACTACTATGATGTCGACGACGA 1140
145                    D  P  H  D  P  Y  R  N  Y  Y  D  V  D  D  E  160

1141 GCGTACGGTCTTTACTTTAGCAGACTGGTACCACACGCCGTCGGAGGCTATCATTGCCAC 1200
160   R  T  V  F  T  L  A  D  W  Y  H  T  P  S  E  A  I  I  A  T    180
```

FIG. 2B

```
1201 CCACGATGTCTTGAAAACgtacgcgttaatccttctagctttctttccttgggtcacttt 1260
180    H  D  V  L  K  T                                              185

1261 ctatcagGATCCCCGACTCGGGTACGATCAACGGCAAAGGCAAATACGATCCTGCTTCGG 1320
185         I  P  D  S  G  T  I  N  G  K  G  K  Y  D  P  A  S         202

1321 CTAACACCAACAACACGACACTCGAGAACCTCTACACTCTCAAAGTCAAACGCGGCAAGC 1380
202  A  N  T  N  N  T  T  L  E  N  L  Y  T  L  K  V  K  R  G  K     222

1381 GGTATCGCCTGAGGATTATCAACGCCTCGGCCATCGCTTCGTTCCGGTTCGGCGTGCAGG 1440
222  R  Y  R  L  R  I  I  N  A  S  A  I  A  S  F  R  F  G  V  Q     242

1441 GCCACAAGTGCACGATCATCGAGGCTGATGGCGTCCTCACCAAACCGATCGAGGTCGATG 1500
242  G  H  K  C  T  I  I  E  A  D  G  V  L  T  K  P  I  E  V  D     262

1501 CGTTTGATATTCTAGCAGGCCAGAGGTATAGCTGCATCgtaagtctacctatgccttgtt 1560
262  A  F  D  I  L  A  G  Q  R  Y  S  C  I                          275

1561 gtggagataagaacctgactgaatgtatgcgctccaatagTTGAAGGCCGACCAAGATCC 1620
275                                            L  K  A  D  Q  D  P   282

1621 TGATTCCTACTGGATAAATGCGCCAATCACAAACGTTCTCAACACCAACGTCCAGGCATT 1680
282   D  S  Y  W  I  N  A  P  I  T  N  V  L  N  T  N  V  Q  A  L   302

1681 GCTAGTGTATGAAGATGACAAGCGTCCTACTCACTACCCCTGGAAGCCGTTTTTGACATG 1740
302   L  V  Y  E  D  D  K  R  P  T  H  Y  P  W  K  P  F  L  T  W   322
```

FIG. 2C

```
1741 GAAGATATCAAATGAAATCATTCAGTACTGGCAGCACAAGCACGGTCGCACGGTCACAA 1800
322   K  I  S  N  E  I  I  Q  Y  W  Q  H  K  H  G  S  H  G  H  K  342

1801 GGGAAAGGGGCATCATCATAAAGTCCGGGCCATTGGAGGTGTATCCGGGTTGAGCTCCAG 1860
342   G  K  G  H  H  H  K  V  R  A  I  G  G  V  S  G  L  S  S  R  362

1861 GGTTAAGAGCCGGGCGAGTGACCTATCGAAGAAGGCTGTCGAGTTGGCTGCTGCACTCGT 1920
349   V  K  S  R  A  S  D  L  S  K  K  A  V  E  L  A  A  A  L  V  349

1921 TGCGGGTGAGGCCGAGTTGGACAAGAGGCAGAATGAGGATAATTCGACTATTGTATTGGA 1980
349   A  G  E  A  E  L  D  K  R  Q  N  E  D  N  S  T  I  V  L  D  361

1981 TGAGACCAAGCTTATTgtaagtcccttaatttttttcggtgtcacggaagctaacccgcg 2040
361   E  T  K  L  I                                               361

2041 taatagCCGTTGGTTCAACCTGGTGCACCGGGCGGCTCCAGACCAGCTGACGTCGTGGTC 2100
361         P  L  V  Q  P  G  A  P  G  G  S  R  P  A  D  V  V  V   379

2101 CCTCTGGACTTTGGCCTCgtatgtggcttcttgttattcgtccggaatgcaaactgattt 2160
379  P  L  D  F  G  L                                             385

2161 gggtgggctatagAACTTTGCCAACGGACTGTGGACGATAAACAATGTCTCCTACTCCCC 2220
385               N  F  A  N  G  L  W  T  I  N  N  V  S  Y  S  P  401

2221 TCCGGATGTCCCTACTCTCCTCAAGATCTTGACCGACAAAGACAAAGTCGACGCTTCTGA 2280
401   P  D  V  P  T  L  L  K  I  L  T  D  K  D  K  V  D  A  S  D  421
```

FIG. 2D

```
2281 CTTgtaggttcctcttcttcttttcaaactagctactgacattaagtgaacgtcagCACG 2340
421   F                                                         T   423

2341 GCCGATGAACACACGTATATTCTTCCAAAGAACCAAGTTGTCGAGTTGCACATCAAGGGA 2400
423   A  D  E  H  T  Y  I  L  P  K  N  Q  V  V  E  L  H  I  K  G   453

2401 CAGGCTTTGGGAATCGTACACCCCCTTCATCTGCATGGCgtacgtctttctcacactgtt 2460
453   Q  A  L  G  I  V  H  P  L  H  L  H  G                       466

2461 ccagctcctattctctaacacactcctgcgatagCATGCGTTCGACGTCGTCCAATTCGG 2520
466                                     H  A  F  D  V  V  Q  F  G   475

2521 CGACAACGCTCCAAACTACGTGAACCCTCCGCGTAGGGATGTAGTAGGCGTAACTGATGC 2580
475   D  N  A  P  N  Y  V  N  P  P  R  R  D  V  V  G  V  T  D  A   495

2581 TGGAGTCCGTATCCAGTTCAGAACCGATAACCCGGGCCCTTGGTTCCTCCATTGgtatgc 2640
495   G  V  R  I  Q  F  R  T  D  N  P  G  P  W  F  L  H  C         513

2641 tcttcatctcccaccgcttgttctttacttatggtttaccttgcgatttagCCACATTGA 2700
513                                                    H  I  D   516

2701 TTGGCACTTGGAAGAAGGATTTGCTAgtaagttattattcctattccgaagcatcggga 2760
516   W  H  L  E  E  G  F  A                                      524

2761 gatgctaaccaagggtgtgttttaagTGGTATTCGCCGAAGCGCCTGAAGATATCAAGAA 2820
524                            M  V  F  A  E  A  P  E  D  I  K  K   536
```

FIG. 2E

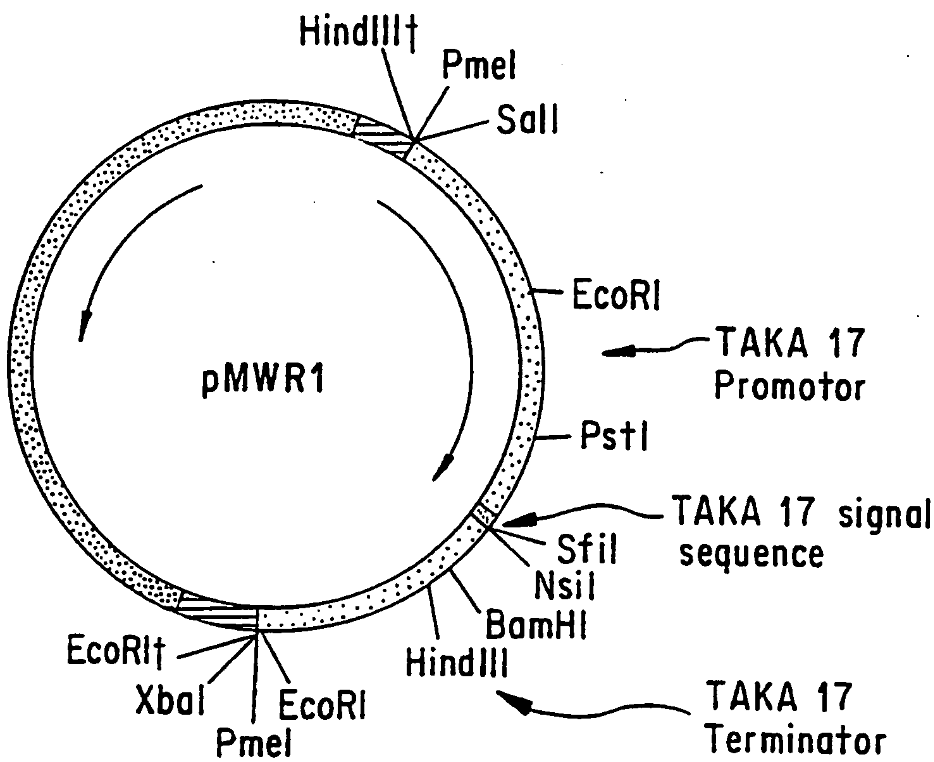
```
2821 AGGCTCTCAGAGTGTCAAGCCTGACGGACAATGGAAGAAACTATGCGAGAAGTATGAGAA 2880
 536   G  S  Q  S  V  K  P  D  G  Q  W  K  K  L  C  E  K  Y  E  K  556

2881 GTTGCCTGAAGCACTGCAGTGAAGTTGCAGTTGTTTCCCATTCGGGAACTGGCTCACTAT 2940
 556   L  P  E  A  L  Q  *                                         562

2941 TCCTTTTGCATAATTCGGACTTTTATTTTGGGACATTATTGGACTATGCATTTGTTTGTC 3000

3001 ACACCGCGGAACTAAGCCGAATTC
```

FIG. 2F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PmeI
SalI
EcoRI
TAKA 17
Promotor
PstI
TAKA 17 signal
sequence
SfiI
NsiI
BamHI
HindIII
TAKA 17
Terminator
EcoRI†
XbaI
EcoRI
PmeI
pMWR1

FIG. 3

```
                 87             96             105            114            123            132
5'  ATG CTT TCT AGC ATT ACC CTC CTA CCT TTG CTC GCT GCG GTC TCA ACC CCC GCC
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
     M   L   S   S   I   T   L   L   P   L   L   A   A   V   S   T   P   A

                141            150            159            168            177            186
    TTT GCT GCC GTC CGC AAC TAT AAG TTC GAC ATC AAG AAC GTC AAT GTC GCT CCC
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
     F   A   A   V   R   N   Y   K   F   D   I   K   N   V   N   V   A   P

                195            204            213            222            231            240
    GAT GGC TTT CAG CGC TCT ATC GTC TCC GTC AAC GGT TTA GTT CCT GGC ACG TTG
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
     D   G   F   Q   R   S   I   V   S   V   N   G   L   V   P   G   T   L

                249            258            267            276            285            294
    ATC ACG GCC AAC AAG GGT GAC ACC TTG CGC ATT AAT GTC ACG AAT CAA CTC ACG
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
     I   T   A   N   K   G   D   T   L   R   I   N   V   T   N   Q   L   T

                303            312            321            330            339            348
    GAC CCT AGT ATG CGT CGT GCC ACA ACG ATT CAT TGG CAT GGA TTG TTC CAA GCT
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
     D   P   S   M   R   R   A   T   T   I   H   W   H   G   L   F   Q   A
```

FIG. 4A

```
                357                 366                 375                 384                 393                 402
ACT ACC GCC GAC GAG GAT GGC CCC GCA TTC GTC ACG CAA TGC CCT ATT GCG CAA
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 T   T   A   D   E   D   G   P   A   F   V   T   Q   C   P   I   A   Q

                411                 420                 429                 438                 447                 456
AAT TTG TCC TAT ACA TAC GAG ATC CCA TTG CGC GGC CAA ACA GGA ACC ATG TGG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 N   L   S   Y   T   Y   E   I   P   L   R   G   Q   T   G   T   M   W

                465                 474                 483                 492                 501                 510
TAT CAC GCC CAT CTT GCG AGT CAA TAT GTC GAT GGA TTG CGA GGC CCT TTG GTC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 Y   H   A   H   L   A   S   Q   Y   V   D   G   L   R   G   P   L   V

                519                 528                 537                 546                 555                 564
ATC TAT GAT CCA AAC GAC CCA CAC AAG TCG CGC TAC GAC GTG GAT GAT GCG AGC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 I   Y   D   P   N   D   P   H   K   S   R   Y   D   V   D   D   A   S

                573                 582                 591                 600                 609                 618
ACA GTA GTC ATG CTT GAG GAC TGG TAC CAT ACT CCG GCA CCC GTT CTA GAA AAG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 T   V   V   M   L   E   D   W   Y   H   T   P   A   P   V   L   E   K
```

FIG. 4B

```
                627             636             645             654             663             672
CAA ATG TTC TCG ACT AAT AAC ACC GCT CTG CTC TCT CCT GTT CCG GAC TCG GGT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 Q   M   F   S   T   N   N   T   A   L   L   S   P   V   P   D   S   G

                681             690             699             708             717             726
CTT ATC AAT GGC AAA GGG CGC TAT GTG GGC GGT CCC GCA GTT CCC CGG TCA GTA
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 L   I   N   G   K   G   R   Y   V   G   G   P   A   V   P   R   S   V

                735             744             753             762             771             780
ATC AAC GTA AAA CGT GGG AAA CGA TAT CGC TTG CGC GTA ATC AAC GCT TCT GCT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 I   N   V   K   R   G   K   R   Y   R   L   R   V   I   N   A   S   A

                789             798             807             816             825             834
ATC GGG TCG TTT ACC TTT TCG ATC GAA GGA CAT AGT CTG ACT GTC ATT GAG GCC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 I   G   S   F   T   F   S   I   E   G   H   S   L   T   V   I   E   A

                843             852             861             870             879             888
GAT GGG ATC CTG CAC CAG CCC TTG GCT GTT GAC AGC TTC CAG ATT TAC GCT GGA
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 D   G   I   L   H   Q   P   L   A   V   D   S   F   Q   I   Y   A   G
```

FIG. 4C

```
        897         906         915         924         933         942
CAA CGC TAC TCT GTC ATC GTT GAA GCC AAC CAA ACC GCC GCC AAC TAC TGG ATT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 Q   R   Y   S   V   I   V   E   A   N   Q   T   A   A   N   Y   W   I

        951         960         969         978         987         996
CGT GCA CCA ATG ACC GTT GCA GGA GCC GGA ACC AAT GCA AAC TTG GAC CCC ACC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 R   A   P   M   T   V   A   G   A   G   T   N   A   N   L   D   P   T

       1005        1014        1023        1032        1041        1050
AAT GTC TTT GCC GTA TTG CAC TAC GAG GGA GCG CCC AAC GCC GAA CCC ACG ACG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 N   V   F   A   V   L   H   Y   E   G   A   P   N   A   E   P   T   T

       1059        1068        1077        1086        1095        1104
GAA CAA GGC AGT GCT ATC GGT ACT GCA CTC GTT GAA GAG AAC CTC CAT GCG CTC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 E   Q   G   S   A   I   G   T   A   L   V   E   E   N   L   H   A   L

       1113        1122        1131        1140        1149        1158
ATC AAC CCT GGC GCT CCG GGC GGC TCC GCT CCC GCA GAC GTT TCC CTC AAT CTT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 I   N   P   G   A   P   G   G   S   A   P   A   D   V   S   L   N   L
```

FIG. 4D

```
                1167                  1176                  1185                  1194                  1203                  1212
GCA  ATT  GGG  CGC  AGC  ACA  GTT  GAT  GGG  ATT  CTT  AGG  TTC  ACA  TTT  AAT  AAC  ATC
---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---
 A    I    G    R    S    T    V    D    G    I    L    R    F    T    F    N    N    I

                1221                  1230                  1239                  1248                  1257                  1266
AAG  TAC  GAG  GCT  CCT  TCG  TTG  CCC  ACG  CTC  TTG  AAG  ATT  TTG  GCA  AAC  AAT  GCG
---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---
 K    Y    E    A    P    S    L    P    T    L    L    K    I    L    A    N    N    A

                1275                  1284                  1293                  1302                  1311                  1320
AGC  AAT  GAC  GCC  GAT  TTC  ACG  CCA  AAT  GAG  CAC  ACT  ATC  GTA  TTG  CCA  CAC  AAT
---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---
 S    N    D    A    D    F    T    P    N    E    H    T    I    V    L    P    H    N

                1329                  1338                  1347                  1356                  1365                  1374
AAA  GTT  ATC  GAG  CTC  AAT  ATC  ACC  GGA  GGT  GCA  GAC  CAC  CCT  ATC  CAT  CTC  CAC
---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---
 K    V    I    E    L    N    I    T    G    G    A    D    H    P    I    H    L    H

                1383                  1392                  1401                  1410                  1419                  1428
GGC  CAT  GTG  TTT  GAT  ATC  GTC  AAA  TCA  CTC  GGT  GGT  ACC  CCG  AAC  TAT  GTC  AAC
---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---
 G    H    V    F    D    I    V    K    S    L    G    G    T    P    N    Y    V    N
```

FIG. 4E

```
         1437                1446                1455                1464                1473                1482
CCG CCA CGC AGG GAC GTA GTT CGT GTC GGA GGC ACC GGT GTG GTA CTC CGA TTC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 P   P   R   R   D   V   V   R   V   G   G   T   G   V   V   L   R   F

         1491                1500                1509                1518                1527                1536
AAG ACC GAT AAC CCA GGC CCA TGG TTT GTT CAC TGC CAC ATT GAC TGG CAC TTG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 K   T   D   N   P   G   P   W   F   V   H   C   H   I   D   W   H   L

         1545                1554                1563                1572                1581                1590
GAG GCT GGG CTC GCA CTT GTC TTT GCC GAG GCC CCC AGC CAG ATT CGC CAG GGT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 E   A   G   L   A   L   V   F   A   E   A   P   S   Q   I   R   Q   G

         1599                1608                1617                1626                1635                1644
GTC CAG TCG GTC CAG CCC AAC AAT GCC TGG AAC CAG CTC TGC CCC AAG TAC GCG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 V   Q   S   V   Q   P   N   N   A   W   N   Q   L   C   P   K   Y   A

         1653                1662
GCT CTT CCT CCC GAT TTG CAG T 3'
--- --- --- --- --- --- --- -
 A   L   P   P   D   L   Q
```

FIG. 4F

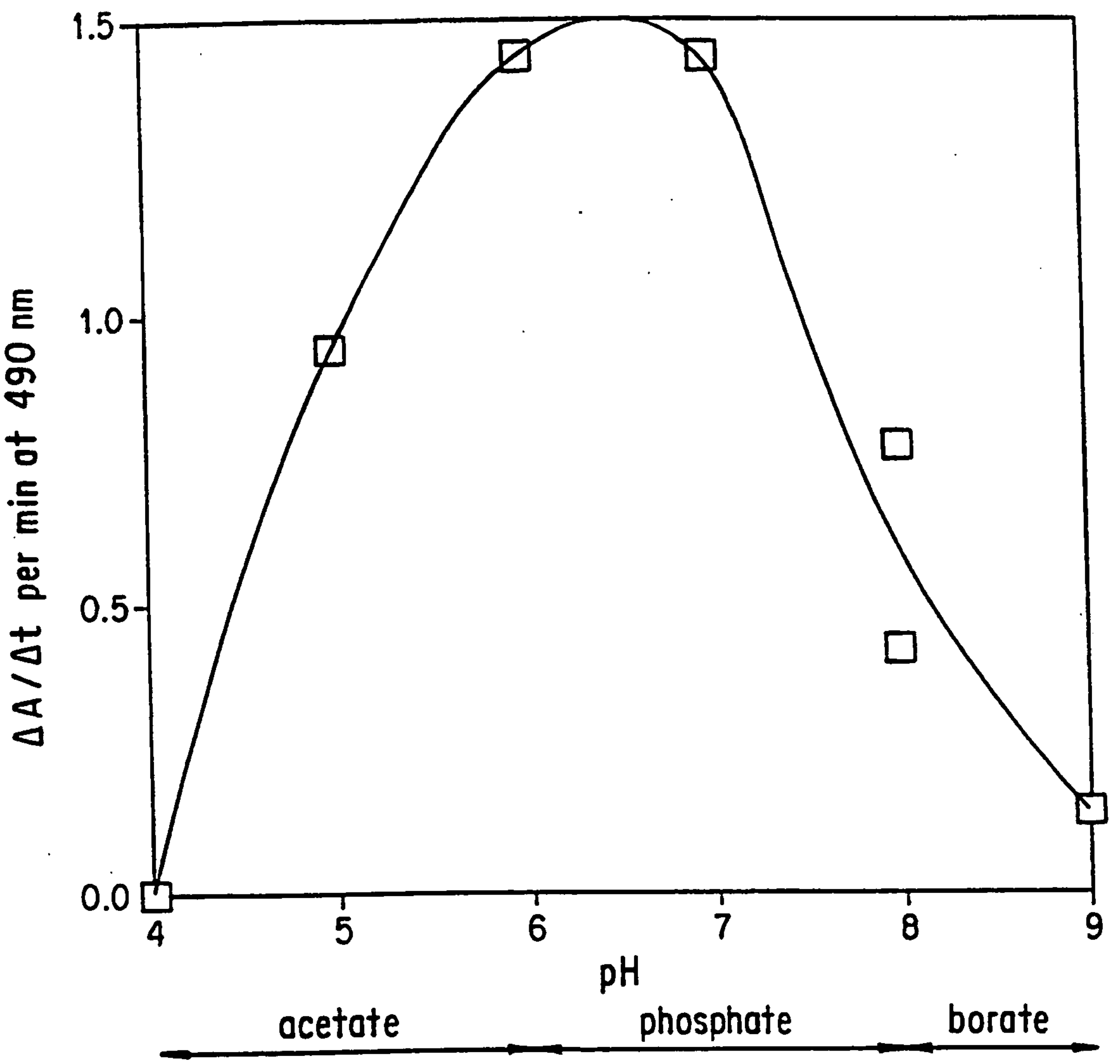
1.5
1.0
0.5
0.0
ΔA/Δt per min at 490 nm
4
5
6
7
8
9
pH
acetate
phosphate
borate

FIG. 5

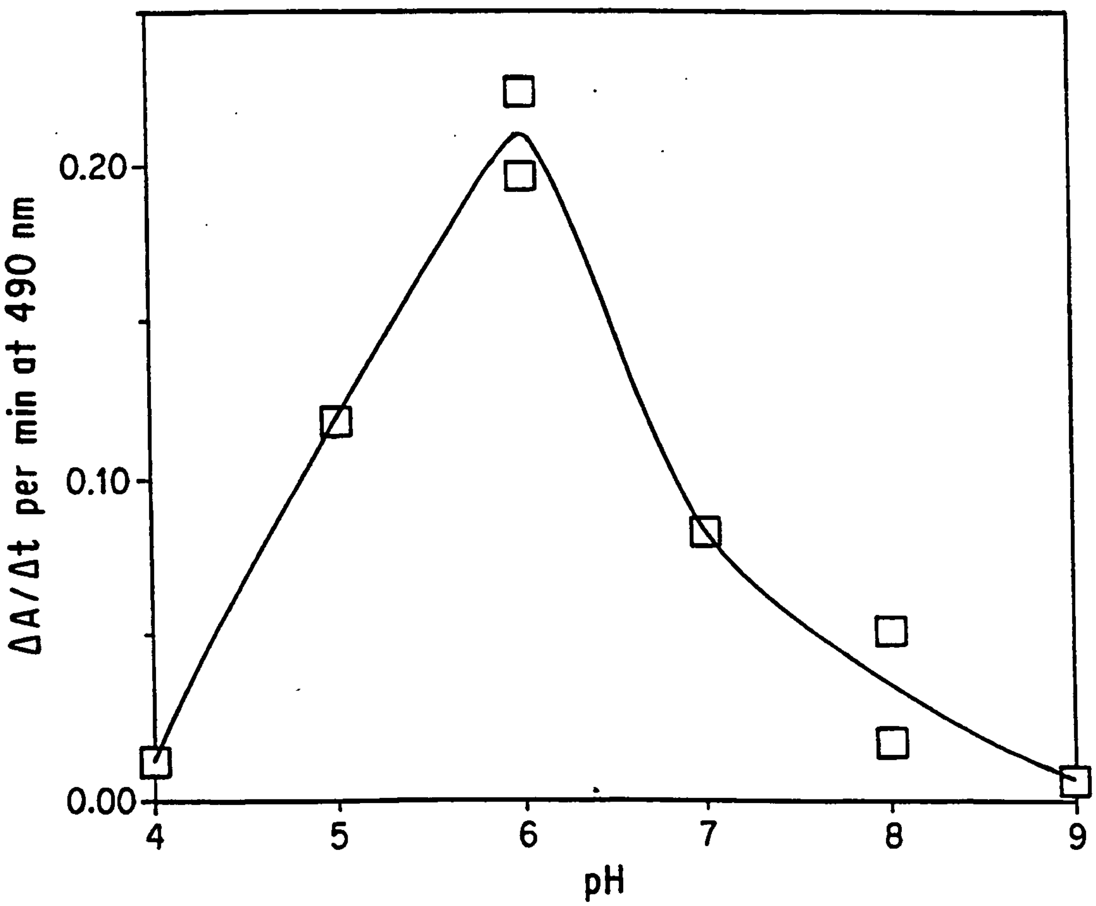

FIG. 6

PCT/US 94/10264

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/53 C12N9/02 C12N15/80 D21C5/00 A61K7/06
C12P7/22 C12N1/19 C09B69/10 //(C12N1/19,C12R1:66)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N D21C A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 January 1995	23.02.95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Delanghe, L

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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		ES-T-	2061857	16-12-94
		JP-A-	3130485	04-06-91
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		JP-A-	6172145	21-06-94